REMARKS

Claims 22, 30, 32, 41-42, 45, 48, 49, 58-66 are pending. As a preliminary matter, correction of inventorship of the instant application has been necessitated due to the cancellation and amendment of the as filed claims and due to the submission of new claims during prosecution of the above identified application. In light of these changes to the claims, Wenhong Li is to be deleted as a co-inventor as outlined in the enclosed Request To Correct Inventorship Under 37 C.F.R. § 1.48(b) and Assignee's Consent to Inventorship Correction Under 37 C.F.R. § 1.48(b).

Rejections under 35 U.S.C. § 112, first paragraph

Claims 61, 62, 65, and 66 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner states that it is unclear where the claimed sequences originated.

Pending claims 61, 62, 65 and 66 are directed to MRI agents comprising a linker and a peptide comprising a caspase sensitive substrate. The specification at pages 21-22 teaches that the nature of the blocking moiety used in the compositions of the invention, depends on the target substance to be detected or visualized. Thus, if the target substance is an enzyme, i.e., a caspase, then blocking moieties can be designed on the basis of the enzyme target substance and the corresponding known characteristics of the enzyme. At the time the application was filed, peptides substrates comprising caspase sensitive sequences were known in the art. See for example:

1) Pennington and Thornberry, 1994, Peptide Research, 7: 72-76 describing YVADAPY (SEQ ID NO. 1; attached herein as Exhibit 1);

2) Thornberry, 1996, British Medical Bulletin, 53: 478-490 describing DEVDG (SEQ ID NO. 2; attached herein as Exhibit 2);

3) Cohen, 1997, Biochem. J., 326: 1-16, a review article disclosing protein substrates of caspases from papers published between the years of 1989 through 1996, including SRVDG (SEQ IN DO. 3); DMQDM (SEQ ID NO. 4), YVHDA (SEQ ID NO. 5), VEIDN (SEQ ID No. 6), DETDS (SEQ ID NO. 7), and DEADG (SEQ ID NO. 8) (attached herein as Exhibit 3).

The general principles governing compliance with the written description requirement provide three alternatives for determining whether there is sufficient written description to inform a skilled artisan that the Applicant was in possession of the claimed invention at the time the application was filed. *See* M.P.E.P § 2163 I and II. M.P.E.P § 2163 II(3)(i)(C) states that the written description requirement can be satisfied if the specification discloses relevant identifying characteristics such that a skilled artisan would be able to predict with a reasonable degree of confidence the structure of the claimed invention.

Applicants' respectfully submit that the specification as filed discloses the requisite identifying characteristics such that a skilled artisan would be able to predict with a reasonable degree of confidence the structure of the claimed invention. Specifically, the specification teaches that MRI agents can be designed comprising blocking moieties that are capable of interacting with caspases. As evidenced by the enclosed Exhibits and the references cited therein, at the time the invention was filed, a number of caspase sensitive cleavage motifs were known to a person skilled in the art. Thus, Applicants respectfully submit that the application as filed satisfies the written description requirement, and request withdrawal of the rejection of claims 61, 62, 65, and 66 under 35 U.S.C. § 112, first paragraph.

Claims 22, 30, 32, 41, 42, 45, 58, 49 and 58-66 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner states that the claims lack written description because they are directed to an unnamed peptide sequence that is cleaved by caspase.

Claims 22, 30, 32, 41, 42, 45, 58, 49 and 58-66 are disclose MRI agents comprising peptides substrates comprising caspase sensitive sequences. As disclosed in Exhibit 3, caspases do not randomly cleave any protein substrate. Rather, protein substrates for caspases comprise recognizable peptide recognition motifs, e.g., DXXD for caspase-3, YVAD for caspase-1, etc. Applicants respectfully submit that pending claims 22, 30, 32, 41, 42, 45, 58, 49 and 58-66 are not directed to unknown peptide sequences that can be cleaved by caspase. As discussed above, the pending claims are directed to peptides comprising caspase sensitive cleavage motifs which were known to a person skilled in the art at the time the application as filed. Applicants respectfully submit that the application as filed satisfies the written description requirement, and request withdrawal of the rejection of claims 22, 30, 32, 41, 42, 45, 58, 49 and 58-66.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 30, 32, 41, 48, 63 and 64 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. Specifically, the Examiner states that it is unclear what Applicant means by the phrase "under conditions whereby said peptide interacts with caspase".

The specification at pages 20-21 teach that what is meant by "capable of interacting with a target substance" is that the blocking moiety has an affinity for the target substance such that blocking moiety will stop blocking or occupying at least one coordination site of the metal ion complex when the target substance is present, thereby freeing at least one coordination site of the metal ion such that rapid exchange of water at this site results in a change in the T_1 of the MRI agent. Thus, as disclosed in pending claims 30, 32, 41, 48, 63 and 64, interaction of the

peptide with a caspase causes a change in the T1 of the MRI agent. Accordingly, Applicants submit that the use of the phrase "under conditions whereby said peptide interacts with caspase" is not indefinite and request withdrawal of the rejection of claims 30, 32, 41, 48, 63 and 64 are rejected under 35 U.S.C. § 112, second paragraph.

Rejections under 35 U.S.C. § 103(a)

Claim 22 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Gries et al., U.S. Patent No. 5, 648,063.

Gries discloses agents for use in NMR and X-ray diagnosis comprising complex salts and a paramagnetic ion to which biomolecules such as peptides and antibodies may be conjugated (see column 3, line 43 through column 4, line 7). Gries *et al.* do not teach or suggest the use of a peptide blocking moiety that is capable of interacting with a target substance such that the blocking moiety stops occupying at least one coordination site of the metal ion complex when the target substance is present, thereby freeing at least one coordination site of the metal ion such that rapid exchange of water at this site results in a change in the T_1 of the MRI agent.

Pending Claim 22 discloses the use of blocking moieties that upon interaction with a target substance stops occupying at least one coordination site of the metal ion complex, thereby freeing at least one coordination site of the metal ion such that rapid exchange of water at this site results in a change in the T_1 of the MRI agent.

To establish a *prima facie* case of obviousness the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991) M.P.E.P. §2143.

Although the Examiner has equated "activated" with the splitting or hydrolyzing of a peptide bond, Applicants specification does not teach MRI agents that are "activated" by the splitting or hydrolyzing of a peptide bond. Rather, Applicants teach MRI agents that are "activated", e.g., "turned on" via an interaction with a target substance such that the T_1 of the agent is changed. Therefore, the requirement of teaching or suggesting all the claim elements has not been met. Applicant respectfully submits the rejection under 35 U.S.C. § 103(a) does not apply to Claim 22 and request withdrawal of the rejection.

Claims 30, 32, 41, 42, 45, 48, 49 and 58-60 are rejected under 35 U.S.C. § 103(a) as being upatentable over Gries et al., U.S. Patent No. 5, 648,063, in view of Zychlinsky et al., U.S. Patent No. 5,972,899

Gries has been discussed above.

Zychlinsky et al teach the use of Shigella IpaB proteins as a means of promoting apoptosis of a cell via the interaction of the IpaB protein with ICE.

As discussed above, the pending claims disclose the use of peptide blocking moieties that upon interaction with a target substance stops occupying at least one coordination site of the metal ion complex, thereby freeing at least one coordination site of the metal ion such that rapid exchange of water at this site results in a change in the T_1 of the MRI agent.

As argued above, Gries *et al.* do not teach or suggest the use of a peptide blocking moiety that is capable of interacting with a target substance such that the blocking moiety stops occupying at least one coordination site of the metal ion complex when the target substance is present, thereby freeing at least one coordination site of the metal ion such that rapid exchange of water at this site results in a change in the T_1 of the MRI agent. This deficiency is not overcome by combining the teachings of Zychlinsky et al., with the teachings of Gries et al.

Accordingly, the requirement of teaching or suggesting all the claim elements has not been met. Applicant respectfully requests withdrawal of the rejection under 35 U.S.C. § 103(a) of claims 30, 32, 41, 42, 45, 48, 49 and 58-60.

Please direct any calls in connection with this application to the undersigned at (415) 781-1989.

Respectfully submitted,

DORSEY & WHITNEY LLP

Dated: 11/10/04

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Filed under 37 C.F.R. § 1.34(a)

Customer Number: **32940**

Exhibit 1

Peptide Research

Synthesis of a Fluorogenic Interleukin-1β Converting Enzyme Substrate Based on Resonance Energy Transfer

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Bachem Bioscience, King of Prussia, PA, and [1]Merck Research Laboratories, Rahway, NJ, USA

ABSTRACT

Interleukin 1β converting enzyme (ICE) is responsible for processing an inactive 31-kDa precursor to the active, mature 17-kDa Il-1β with cleavage occurring between the Asp^{116}-Ala^{117} amide bond. We have prepared a peptide substrate that contains the protease cleavage site situated between two fluorophores located at the termini of the molecule. Upon cleavage of DABCYL-Tyr-Val-Ala-Asp-Ala-Pro-Val-EDANS (DABCYL-ICE-EDANS), an increase in fluorescence is observed at the EDANS emission wavelength of 490 nm, permitting a continuous assay of ICE that is useful in the screening of inhibitory compounds. The K_m and k_{cat} results for hydrolysis of DABCYL-ICE-EDANS by ICE were 11.4 ± 1.6 μM and 0.79 ± 0.4 s^{-1}. The second order rate constant for hydrolysis of this substrate (k_{cat}/K_m = 7.0 + 1.3 × 10^4 M^{-1} s^{-1}) is comparable to that for the cleavage of the previously described fluorogenic substrate, Ac-Tyr-Val-Ala-Asp-AMC (6.4 × 10^4 M^{-1} s^{-1}).

INTRODUCTION

The development of a rapid enzyme assay procedure is essential in order to generate data to elucidate the mechanism, to determine the specificity and to prepare potent inhibitors for the enzyme. Spectroscopic methods are among the fastest to generate data in this process. Our interests have been centered on the application of resonance energy transfer properties to a small peptide substrate containing the cleavage site specific for the enzyme responsible for the proteolytic activation of interleukin-1β, an important mediator of inflammation.

Interleukin-1β converting enzyme (ICE) is a heterodimeric cysteine protease (15) that catalyzes the conversion of the inactive 33 kDa or 31 kDa Il-1β precursor to the 17.5 kDa mature, biologically active form (5,9). The unique cleavage site appears to be evolutionarily conserved in all known Il-1β molecules (13). This cleavage site occurs between Asp 116 and Ala 117 in human Il-1β (2,10). Cleavage of the precursor by ICE is dependent upon an aspartic acid residue in the P_1 position (3,4). However, ICE appears to be very selective in that it does not cleave other proteins, including Il-1α, that contain multiple Asp-X bonds (3).

A series of peptide substrates has been reported to be processed by the enzyme with reasonable efficiency (15,18). Many of these substrates required HPLC-based assay procedures, which are very time consuming and slow. One fluorogenic substrate (Ac-Tyr-Val-Ala-Asp-AMC, where AMC represents aminomethylcoumarin) was described and determined to be very effective in a continuous fluorescence assay (15). In this report, we apply the fluorescence resonance energy transfer (FRET) principle to a peptide substrate specific for ICE.

MATERIALS AND METHODS

All reagents and solvents were of reagent grade and used without any further purification. Amino acid derivatives and resins were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). 4-(4′-Dimethylaminophenylazo)benzoic acid (DABCYL) was obtained from TCI America (Portland, OR, USA). 5-[(2-Aminoethyl)-amino]-naphthalene-1-sulfonic acid (Na salt) (EDANS) and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) were purchased from Sigma Chemical (St. Louis, MO, USA). Benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent) was purchased from Richelieu Biotechnologies (Quebec, Canada).

Peptide Synthesis

The C-terminal fragment was prepared using a Boc strategy using classical peptide synthesis with EDC (water-soluble carbodiimide) and 1-hydroxybenzotriazole (HOBT) mediated couplings. Boc-Val-OH was derivatized with EDANS in the presence of EDC and HOBT. Following purification by reversed-phase HPLC (RPHPLC), Boc-Val-EDANS was treated with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) to remove the Boc group. The TFA and DCM were removed *in vacuo*, the TFA salt was neutralized with triethylamine and the Val-EDANS was coupled in solution to Boc-Ala-Pro-OH. The resulting protected peptide (Boc-Ala-Pro-Val-EDANS) was treated with 50% TFA in DCM and concentrated *in vacuo*. The TFA salt was neutralized yielding Ala-Pro-Val-EDANS. This peptide represents the C-terminal cleavage product following processing by ICE of the complete peptide substrate.

The N-terminal cleavage product was synthesized by an Fmoc-based solid-phase strategy employing Sasrin™ resin (Bachem Feinchemikalien, Bubendorf, Switzerland). DABCYL-Tyr(tBu)-Val-Ala-Asp(OtBu)-OH was synthesized stepwise starting with Fmoc-Asp(OtBu)-Sasrin resin. DABCYL coupling (6 eq.) was mediated by diisopropylcarbodiimide/HOBT in *N*-methylpyrrolidinone. This coupling was allowed to proceed overnight. The protected peptide fragment (3 mmol) was released from the resin by treatment with 0.5% TFA in DCM (7,8). The protected peptide fragment solution was immediately neutralized with 1.2 eq. of pyridine and concentrated *in vacuo*. The protected peptide fragment was redissolved in dimethylformamide (DMF). This fragment was activated by adding 3.2 mmol of BOP reagent and 5 eq. of diisopropylethylamine (DIEA) to the protected peptide fragment solution. Following 5 min activation, 0.5 mmol of Ala-Pro-Val-EDANS was added and allowed to couple at room temperature. The progress of the reaction was followed by RPHPLC and determined to be complete after 48 h, at which point the peptide was precipitated into H_2O. The peptide fragment was redissolved in 50% TFA in DCM to deprotect the side-chain functionalities of Asp and Tyr. The TFA and DCM were removed *in vacuo* and the peptide was redissolved in neat TFA and loaded onto a RPHPLC C_{18} column (Vydac; The Separations Group, Hesperia, CA, USA). Purification required a two-step procedure of aqueous triethylamine phosphate (TEAP) (12) pH 2.3 vs. acetonitrile (ACN). A second TEAP run was performed using a shallower gradient by employing aqueous TEAP vs. 60% ACN/40% aq. TEAP. Pure fractions were pooled together and desalted by RPHPLC employing 0.1% TFA in H_2O vs. 0.1% TFA in ACN. Following final purification and lyophilization, 186 mg of red solid product was obtained. Analysis of DABCYL-Tyr-Val-Ala-Asp-Ala-Pro-Val-EDANS: Compound dissolved in DMF had a dark amber color. Mass spectrum (FAB): $(M+H)^+$ = 1234, $(M+Na)^+$ = 1256. Amino acid composition: Asx (1) 1.05, Pro (1) 0.96, Ala (2) 2.06, Val (2) 1.92, Tyr (1) 1.01.

Optical purity of the amino acid residues in the peptide was obtained from C.A.T. GmbH & Co. (Tubingen, Germany). The sample was hydrolyzed in 6 *N* DCl/D_2O, whereby racemization was accompanied by deuterium exchange in the α-C position. After exchange of the labile deuterium for hydrogen, the amino acids were derivatized (esterification and perfluoracylation) and gas-chromatographically separated on a Chirasil-Val capillary using EI-SIM-mass spectroscopy for detection. The relative amounts of D- and L-enantiomers originally present in the sample were determined by monitoring the non-deuterated molecular ions.

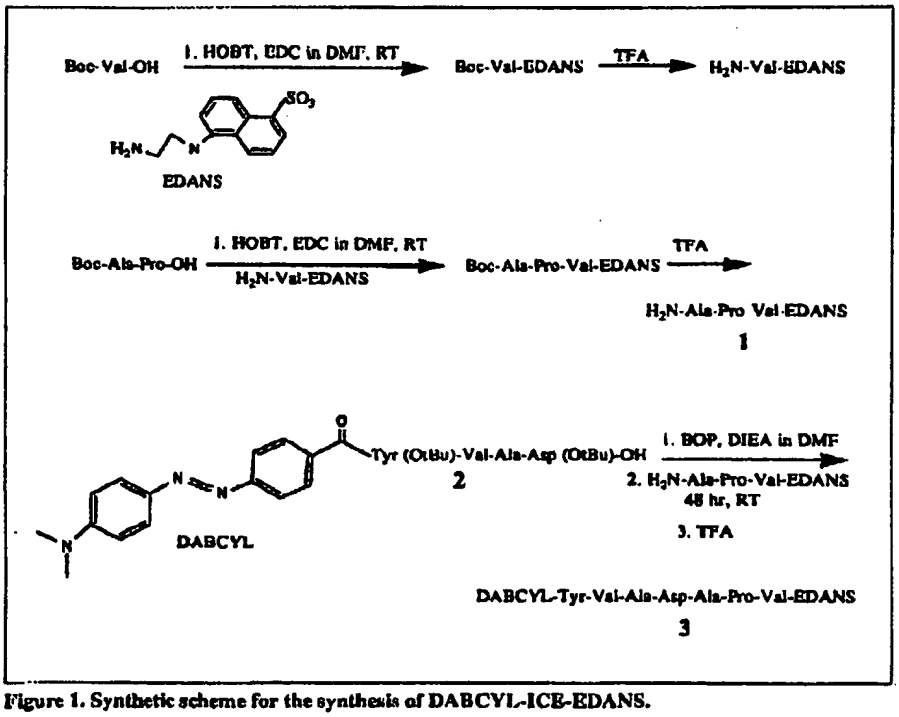

Figure 1. Synthetic scheme for the synthesis of DABCYL-ICE-EDANS.

Kinetic Methods

The enzyme employed in these studies was purified to homogeneity from THP.1 cells by affinity chromatography as previously described (15). Concentration of active enzyme was determined by titration with an active site-directed, irreversible inhibitor (14), the peptide acyloxymethylketone (Ac - Tyr - Val - Ala - Asp - O - CO - [2,6 -$(CF_3)_2$]) Ph (16). A 10 mM stock solution of the substrate sample was prepared in DMF. Cleavage of DABCYL-ICE-EDANS was monitored continuously in a Gilford Fluoro IV spectrofluorometer (Oberlin, OH, USA) using an excitation wavelength of 340 nm and an emission wavelength of 490 nm (6). Standard assay conditions were 100 mM Hepes, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml OVA at 25°C. To measure the kinetic parameters, K_m and k_{cat}, enzyme (10.7 nM) was added to the reaction mixtures containing the substrate (2–100 μM) and buffer in a total volume of 500 μl using a 40-mm × 12.5-mm × 12.5-mm cuvette. The spectrofluorometer was calibrated so that complete hydrolysis of the 5 μM substrate resulted in 100% relative fluorescence. Data were transferred via RS232 interface for analysis by a Sun Microsystems SPARCstation 10 Workstation (Mountain View, CA). Kinetic constants were computed by direct fits of the data to the Michaelis-Menton equation using a nonlinear least squares analysis computer program (NLIN) developed in this laboratory (N.A.T.). This program, when furnished with the equation and its partial derivatives with respect to each unknown parameter, uses Marquardt algorithm to converge on the best estimates of the parameters and provides the standard error of each estimate.

RESULTS AND DISCUSSION

Recently, the resonance energy transfer principle was adapted to a

retroviral substrate for HIV protease (6,17). The substrate incorporated a DABCYL (4-[4'-dimethylaminobenzeneazo]benzoic acid) as a quenching acceptor group and EDANS (5-[2'-aminoethyl]-aminonaphthlene as the donor fluorophore. These two chromophores are ideal for derivatizing potential enzyme substrates because of the excellent spectral overlap of the EDANS emission with the DABCYL absorption band, which leads to very efficient energy transfer (6). Additionally, EDANS is a relatively long-lived donor that helps to suppress residual substrate fluorescence, as the resonance energy transfer is enhanced by diffusion of the donor and the acceptor (6). Furthermore, the substrate sequence was ideal for derivatization, as there were no reactive side-chain functionalities to complicate the reaction. Thus, solid-phase synthesis of the substrate (DABCYL-Gaba-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-OH) was easily accomplished, including attachment of the DABCYL moiety. The peptide was cleaved and deprotected, and subsequently derivatized with the EDANS group at the C-terminal carboxyl group in solution (11,17).

For ICE, selection of a seven-residue peptide sequence to derivatize with the DABCYL and EDANS was based upon the substrate series studied in the initial report by Thornberry et al. (15). The heptapeptide sequence Tyr-Val-Ala-Asp-Ala-Pro-Val contains the necessary cleavage site of Asp-Ala.

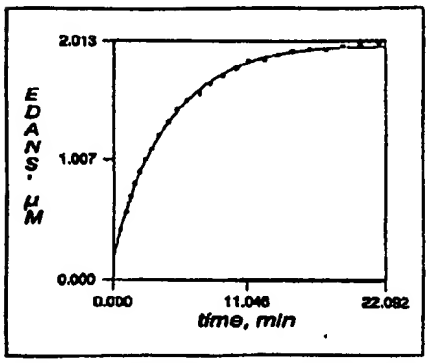

Figure 2. Hydrolysis of DABCYL-ICE-EDANS. Enzyme (57 nM) was added to reaction mixtures containing micromolar substrate concentrations under standard reaction conditions. (See Materials and Methods.) The data were fit by non-linear regression to 1st-order rate equation to obtain a value for the observed 1st-order rate constant, k_{obs} = 0.224 min^{-1}, corresponding to a 2nd-order rate constant for hydrolysis $k_{obs}/[Enz] = k_{cat}/K_m$, of 6.5×10^4 M^{-1} s^{-1}.

Assuming an extended conformation for this peptide, the separation distance of the donor–acceptor (D/A) pair is expected to be 22.6 Å.

Synthesis of the ICE substrate introduces a secondary reactive carboxyl group at an Asp residue. This functionality must be masked in order to obtain incorporation of the EDANS exclusively at the C-terminal carboxyl group and ultimately deprotected to yield the free side chain carboxyl group. In an initial attempt, a standard solid-phase assembly of the entire sequence by a Boc-based strategy incorporating Boc-Asp-(OFm)-OH resulted in a complicated mixture following HF cleavage. The synthesis was further complicated upon attempting to perform a solution coupling of the EDANS to the C-terminal carboxyl and subsequent treatment with piperidine to remove the fluorenylmethyl protecting group from the Asp side chain. Thus, a solution strategy was adapted to eliminate these complications. Figure 1 outlines the synthetic route for the fluorescent ICE substrate (DABCYL-Tyr-Val-Ala-Asp-Ala-Pro-Val-EDANS) (DABCYL-ICE-EDANS). By adopting this solution strategy, each of the two cleavage products were synthesized and used for verification of the cleavage site.

Initial condensation attempts utilizing DCC/HOBT activation were unsuccessful, so BOP reagent in the presence of DIEA was used. This activation method effectively condensed the two fragments in 36 to 48 h. As it was not possible to condense the fragments through a Gly residue, we elected to condense through the Asp residue. Since racemization values

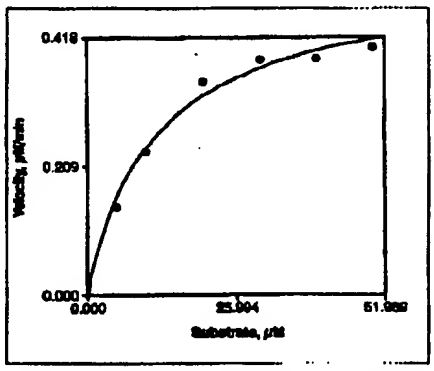

Figure 3. Standard velocity vs. substrate concentration profile for the hydrolysis of DABCYL-ICE-EDANS. Assay conditions are described in the Materials and Methods section of this report.

were not readily available for Asp (OtBu), we used literature reports that had determined that Asp(OBzl) was among the amino acids least sensitive to racemization using a BOP protocol, making it one of the best assembly points (1). Following the condensation, we determined the amount of D-enantiomer of Asp to be 3.4%. This value is in agreement with that determined for Asp(OBzl).

In order to obtain complete dissolution of the DABCYL-ICE-EDANS for enzyme assay purposes, a stock solution was prepared in DMF because of its insolubility in purely aqueous solutions. Solutions of DABCYL-ICE-EDANS of >50 mg/ml in DMF are possible; however, when an aliquot of this solution was diluted 10-fold into the aqueous assay buffer, the DABCYL-ICE-EDANS slowly began to precipitate from solution. Furthermore, a concentration of 5 mg/ml is more than 25-fold higher than the useful active working concentration range of 10 to 20 µg/ml (ca. 10–20 µM).

The substrate DABCYL-ICE-EDANS is cleaved specifically at the Asp-Ala amide bond by ICE. Cleavage of the substrate results in a steady increase in fluorescence intensity, which permits continuous monitoring of the enzyme activity. Exhaustive enzymatic conversion of substrate to product results in an enhancement in fluorescence of >20-fold. A typical progress curve, measured under conditions where the substrate concentration is below K_m (see below), is shown in Figure 2, where the solid line is theoretical for a second-order rate constant for hydrolysis, k_{cat}/K_m of 6.5×10^4 M^{-1} s^{-1}.

To determine the K_m and k_{cat} for hydrolysis of DABCYL-ICE-EDANS by ICE, initial velocities were measured at several substrate concentrations. The fluorescence due to product deviates from linearity in the presence of relatively high substrate concentrations (7% at 40 µM). [A reviewer of this manuscript has informed us that the observed loss of fluorescence at high substrate concentration is due to an instrument measurement artifact termed the "inner filter effect," which must be empirically determined for each spectrofluorometer.] Consequently, velocities were corrected for this loss of fluorescence prior to analysis. Velocities and substrate concentrations were fit to the Michaelis-Menton equation

by nonlinear regression. The results are shown in Figure 3, where the solid line is theoretical for values of K_m and k_{cat} of 11.4 ± 1.6 μM and 0.7 ± 90.4 s^{-1}. The second-order rate constant for hydrolysis calculated from this experiment, k_{cat}/K_m = 7.0 ± 1.3 × 10^4 M^{-1} s^{-1}, agrees well with that determined above and is comparable to that for cleavage of the previously described fluorogenic substrate, Ac-Tyr-Val-Ala-Asp-AMC (6.4 × 10^4 M^{-1} s^{-1}) (14).

Cleavage of the substrate can also be monitored by RPHPLC as shown in Figure 4. In this HPLC trace, one of the cleavage product peaks elutes significantly earlier than the uncleaved substrate peak. The earliest eluting peak represents the C-terminal cleavage product H-Ala-Pro-Val-EDANS. The middle peak, which elutes just in front of the unprocessed substrate, represents the DABCYL-Tyr-Val-Ala-Asp-OH product, and the late eluting peak is uncleaved substrate.

Both Ac-Tyr-Val-Ala-Asp-AMC and DABCYL-ICE-EDANS are excellent substrates for assaying ICE activity. Because of the small amount of D-enantiomer in DABCYL-ICE-EDANS, total conversion of the substrate to product is not possible, as ICE requires an L-Asp at the P_1 position. Thus, the resulting kinetic properties K_m and k_{cat} will be slightly (± 3%) lower and higher, respectively.

Both Ac-Tyr-Val-Ala-Asp-AMC and DABCYL-ICE-EDANS are relatively stable compounds and have similar kinetic properties. The main advantage that the DABCYL-ICE-EDANS offers over the Ac-Tyr-Val-Ala-Asp-AMC is the ability to investigate potential interactions at the P'_1-P'_3 positions, which can yield very useful information about the subsite preferences of the S'_1, S'_2 and S'_3 of ICE.

It appears that the positioning of the D/A pair does not significantly affect the binding or cleavage of the peptide recognition sequence for ICE. We have shown that resonance energy transfer can be effectively used to assay ICE. This type of assay, because of its simplicity and speed, may be easily adapted to the large-scale screening of potential inhibitors with the use of a microplate and a 96 well microplate fluorescence reader.

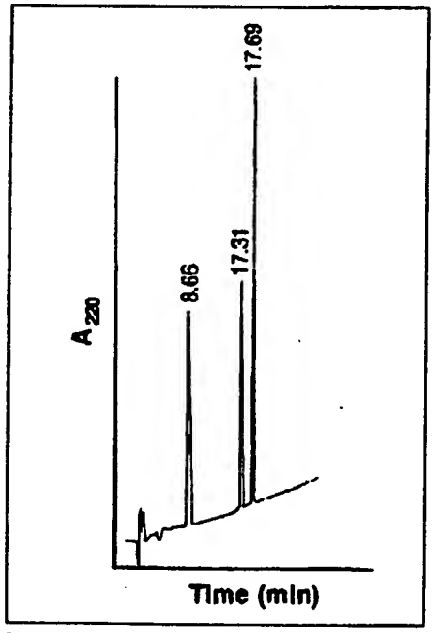

Figure 4. RP-HPLC trace of the cleavage of the DABCYL-ICE-EDANS (17.69 min) and the two cleavage products. RP-HPLC gradient conditions: Vydac C_{18}-silica column employing a linear gradient from 5% to 50% 0.1% TFA in ACN vs. 0.1% TFA in H_2O in 25 min at a flow rate of 1.5 ml/min.

ACKNOWLEDGMENTS

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The caspase family of cysteine proteases

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The discovery that CED-3, the product of a gene necessary for programmed cell death in the nematode *Caenorhabditis elegans*, is related to the mammalian cysteine protease interleukin-1β converting enzyme (ICE/caspase-1) has led to intense interest in the role of proteases in apoptosis. It is now clear that at least some members of the caspase (ICE/CED-3) family, which at present includes ten homologues of human origin, are essential components of an evolutionarily conserved pathway of apoptosis. These enzymes appear to be involved in both the initial signaling events and the downstream proteolytic cleavages that result in the apoptotic phenotype. Selective macromolecular and peptide-based inhibitors attenuate apoptosis in whole cells, suggesting that one or more of these enzymes will be suitable targets for therapeutic intervention in diseases resulting from inappropriate cell death.

Historical perspective

In the nematode *Caenorhabditis elegans* (*C. elegans*), 131 of the 1090 somatic cells formed during development of an adult undergo programmed cell death (for review, see[1]). Elegant genetic studies have established that two genes, *ced-3* and *ced-4*, are required for this cell death, in that deletions or mutations in either gene result in the survival of all of the cells that normally die[2]. Late in 1993, CED-3 was shown to be homologous to interleukin-1β converting enzyme (ICE/caspase-1)[3], a cysteine protease originally identified as the enzyme responsible for the processing of the pro-inflammatory cytokine, pro-interleukin-1β (pro-IL-1β)[4]. This finding, together with the observation that a negative regulator of apoptosis in mammals, Bcl-2, is homologous to a repressor of cell death found in *C. elegans*[5], strongly suggested that at least some of the mechanisms of cell death are evolutionarily conserved, and that proteases are important mediators in this process.

When caspase-1 was first purified, cloned and sequenced in 1992[4,6], it was found to be unrelated to any known protein. The finding that it is related to CED-3 prompted an intense search for other mammalian homologs and, to date, ten proteases of human origin have been

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THE CASPASE FAMILY OF CYSTEINE PROTEASES

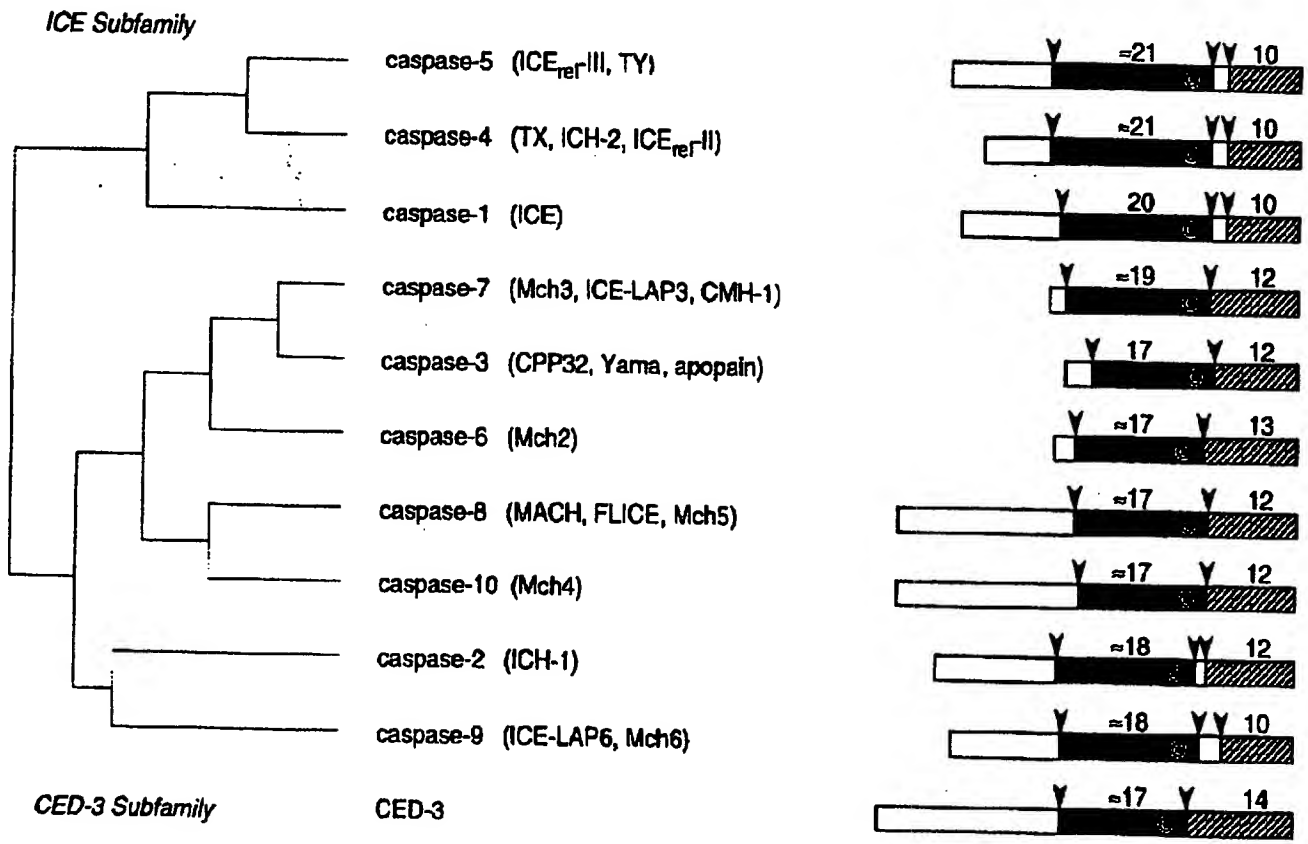

Fig. 1 The caspase family of cysteine proteases. Aliases for the proteases are shown in parentheses. The phylogenic relationships were determined using the PILEUP algorithm of the Genetics Computer Group (version 8.1; gap weight=3.0; gap length weight=0.1). (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, WI 53711, USA). Known members of the caspase family include the C. elegans CED-3 and ten enzymes of human origin. The proteins can be tentatively grouped into two subfamilies, based on their sequence homologies to caspase-1 and CED-3. All family members are synthesized as proenzymes (shown) that are proteolytically processed at Asp-X sites (indicated by arrows) to generate the heterodimeric, mature enzymes.

identified (Fig. 1) (for review, see[7]). A unified nomenclature for these enzymes has recently been adopted, using the term 'caspase' as a root for serial names[8]. A phylogenetic analysis of the caspases suggests that these enzymes may be grouped into two, possibly three, subfamilies.

It is now clear that at least some of these enzymes, particularly those most highly related to CED-3, are essential mediators of mammalian apoptosis. First, macromolecular and peptide-based inhibitors of caspases prevent cell death in both *in vitro* and *in vivo* models of apoptosis (for examples, see[9–12]). Inhibition is observed in many cell types and in response to diverse stimuli suggesting that these enzymes are components of an essential, conserved cell death mechanism. Second, caspases appear to be responsible for the hydrolysis of several key homeostatic, repair and structural proteins that are cleaved during apoptosis (for review, see[7]). Third, post-translational activation of several caspases has been shown to correlate with the appearance of the apoptotic phenotype[13–15]. Finally, and most compelling, mice deficient in caspase-3 have a defect in the apoptosis that occurs during brain development[16]. These animals die at 1–3 weeks of age and display profound abnormalities of the nervous system. Surprisingly, there are no obvious defects in the development of the other major organs, or in thymocyte apoptosis, even though caspase-3 is normally expressed in these tissues, suggesting that caspases have redundant functions in some cell types.

In contrast to the phenotype observed with caspase-3 deficient animals, mice unable to produce caspase-1 have no major defects in apoptosis, indicating that this family member does not play a central, non-redundant role in this process[17,18]. However, these mice do have a defect in IL-1β production, and are resistant to LPS-induced endotoxic shock, confirming a role for this protease in inflammation. Taken together, studies to date indicate that caspases have diverse functions, with members of this family playing essential roles in both inflammation and apoptosis.

Distinguishing properties of caspases

The two best characterized members of the caspase family are caspase-1 and caspase-3, the latter being the human enzyme most closely related to CED-3. Both have been purified from natural sources, cloned, and their structures determined by X-ray crystallography[4,11,19–21]. The results of these studies, together with a comparison of the primary sequences of all of known caspases, suggest that the following are distinguishing structural and catalytic properties of these enzymes.

First, the mature enzymes are heterodimers composed of two subunits with approximate molecular weights of 10 and 20 kDa. The two subunits are intimately associated to form a single catalytic domain in which both contribute key residues to the active site. In analogy with other cysteine proteases, the active site contains a catalytic diad composed of a cysteine sulphydryl group in close proximity to a histidine imidazole group, which is presumed to function as a general acid/base during substrate hydrolysis. The oxyanion of the tetrahedral intermediates formed during catalysis appears to be stabilized through hydrogen bonding interactions in what is typically referred to as an 'oxyanion hole'. In the crystal structures of caspase-1 and caspase-3 in complex with peptide-based inhibitors, two heterodimers associate to form a tetramer, and it has been proposed that this is the active form of the enzyme in solution.

Second, all of these enzymes appear to have a near absolute requirement for aspartic acid in the P_1 position in both macromolecular and peptide substrates. Caspase-1 catalyzes the cleavage of the inactive 31 kDa human pro-IL-1β at Asp_{116}–Ala_{117} to generate the 17.5 kDa mature, biologically active cytokine. Substitution of the P_1 position with any other natural amino acid in pro-IL-1β and peptide-based substrates results in more than a 100-fold decrease in k_{cat}/K_m[22,23]. The crystal structures of caspase-1 and caspase-3 define the four residues that are responsible for this selective interaction (Arg_{179}, Gln_{283}, Arg_{341}, Ser_{347}), and all of these are conserved among all known family members. It is intriguing that these cysteine proteases, and the cytotoxic lymphocyte-derived serine protease granzyme B, which also functions in apoptosis, are the only mammalian proteases known to have this specificity.

Third, in contrast to the apparent similarities between the S_1 subsites of known caspases, the chemical nature and geometry of other subsites (most notably S_4) do not appear to be conserved among all caspases, providing a rationale for the distinct macromolecular specificities, and hence functions, of these enzymes. For example, studies with both peptide and protein substrates indicate that caspase-1 prefers large, hydrophobic amino acids in the P_4 position, while in caspase-3 all of the endogenous substrates that have been identified to date contain an aspartic acid in this position. This P_4 aspartic acid appears to be required for efficient hydrolysis by caspase-3, in that replacement of this residue with any other amino acid results in >100-fold decrease in k_{cat}/K_m. A comparison of S_4 in the crystal structures of caspases 1 and 3 reveals profound differences in both the geometry and the chemical composition of this subsite that explain their distinct specificities. For example, S_4 in caspase-1 is a shallow depression that easily accommodates a hydrophobic side chain. In contrast, in caspase-3 S_4 is a narrow pocket containing several side chains that serve to stabilize the aspartic acid.

Many of the residues in caspase-3 that are responsible for its stringent specificity for Asp in S_4 are conserved among closely related homologs, suggesting that at least some members of the caspase family will have similar specificities. This is consistent with the hypothesis that some of these enzyme have redundant functions.

Finally, as shown in Figure 1, all of these enzymes are synthesized as proenzymes from which both subunits of the heterodimer are derived by proteolytic processing[4,11]. The genes for these proenzymes also encode an N-terminal peptide of variable length (approximately 3–13 kDa), and in some cases a linker peptide between the two subunits, both of which are removed during proteolytic activation. Both the large and small subunits of the heterodimer are flanked by Asp-X bonds, suggesting that activation is either autocatalytic, or mediated by an enzyme with a similar specificity. These alternate mechanisms for activation are considered below.

Inhibitors of caspases

Several macromolecular and peptide-based inhibitors of caspases have been identified and used to secure a role for at least some of these enzyme in apoptosis. Two naturally occurring protein inhibitors have been described. Cytokine response modifier A (CrmA) is a 38 kDa serpin from cowpox virus that appears to facilitate infection through both inhibition of the host inflammatory response, and inhibition of apoptosis[24]. CrmA has been evaluated as an inhibitor of both caspase-1 and caspase-3, and found to exhibit very different potencies against the two enzymes[11,25]. It is a potent inhibitor of caspase-1 (K_i=4 pM, $k_{on}=1.7\times10^7\ M^{-1}s^{-1}$) and a weak caspase-3 inhibitor ($K_i > 100$ nM, $k_{on} < 1\times10^4\ M^{-1}s^{-1}$). The poor inhibition observed with caspase-3 can be explained by the stringent specificity of this enzyme for Asp in S_4, as CrmA requires recognition at the sequence LVAD for inhibition. The target(s) for CrmA inhibition during apoptosis has not been established, but it is likely that its mechanism involves inhibition of these proteases, because, as mentioned above, cleavage after Asp is uncommon among proteases.

Another viral gene product, a 35 kDa protein from baculovirus (p35), also appears to attenuate apoptosis through inhibition of the caspases. Expression of p35 has been shown to prevent cell death in insect cells, *C. elegans*, and in mammalian systems of apoptosis[26]. It has been shown to be an irreversible inhibitor of several family members, including caspases 1, 2, 3, 4 and CED-3[27,28]. The ability of this protein to inhibit cell death

induced by a variety of stimuli in phylogenetically diverse organisms is further evidence for an evolutionarily conserved role for the caspases.

Turning to small molecule inhibitors, several classes of reversible and irreversible peptide-based inhibitors have been designed using strategies that have proven successful for inhibition of other cysteine proteases[29,30]. Reversible inhibitors include aldehydes, nitriles and ketones. Irreversible inhibitors are of the general structure Peptide–CO–CH_2–X, where X is a halide ion (chloromethylketones, fluoromethylketones), $-N_2$ (diazomethylketones), –OCOR ((acyloxy)methylketones), or –OR (α-(pyrazoloxy)methylketones and (phosphinyloxy)methylketones).

The peptide moiety of these inhibitors determines their selectivity for particular caspases. For example, the tetrapeptide aldehyde based on the pro-IL-1β cleavage site, Ac–YVAD–CHO, is a potent inhibitor of caspase-1 (K_i=0.76 nM), and a weak inhibitor of caspase-3 (K_i=10,000 nM)[11]. This reflects the inability of caspase-3 to tolerate hydrophobic residues in S_4. In contrast, the aldehyde containing the PARP cleavage site, Ac–DEVD–CHO, is a relatively potent inhibitor of both caspase-1 and caspase-3, with a 49-fold preference for caspase-3 ($K_{i,caspase\text{-}3}$=0.35 nM, $K_{i,caspase\text{-}1}$=17 nM)[7]. The reduced selectivity of this inhibitor is an indication of the promiscuity of the S_4 subsite of caspase-1, which prefers hydrophobic amino acids, but accepts many others (including aspartic acid).

As mentioned above, these aldehyde inhibitors, and several other peptide-based inhibitors of these enzymes, have been shown to be effective at preventing apoptosis in diverse models of apoptosis. However, these compounds are not useful as probes of biological function for particular family members because extremely high concentrations (>10 μM) are generally required for efficacy, presumably because of poor cell penetration.

Roles in apoptosis

Current evidence suggests that caspases are essential components of a proteolytic cascade that is triggered in response to a death stimulus, having roles in both the regulation and execution stages of apoptosis. For example, in apoptosis induced by Fas ligand, caspase-8 has been implicated in upstream signaling events, while caspase-3 and closely related homologs appear to be involved in the effector phase of apoptosis. The phenotype of caspase-3 deficient mice strongly suggests that at least some caspases have redundant functions within mammalian cells, presumably in both stages of apoptosis. The proposed biochemical

roles of these enzymes in apoptosis are described in detail below, and summarized in a putative model for Fas-induced apoptosis (Fig. 2).

Cleavage of homeostatic and structural proteins

The identification of potential endogenous substrates for the caspases has provided important clues to their molecular role(s) in apoptosis. Proteins that are known to be cleaved during apoptosis generally fall into two major groups: (i) catalytic proteins functioning in homeostatic pathways; and (ii) those involved in organization and maintenance of cell structure (for review, see[7]). In every case, hydrolysis occurs after aspartic acid, suggesting the involvement of a caspase, or an enzyme with similar specificity (e.g. granzyme B).

As summarized in Table 1, several proteins that are believed to be involved in maintenance of normal cell function and repair processes are cleaved during apoptosis. It has been suggested that crippling of these pathways may be a fundamental feature of apoptosis, to ensure the rapid irreversibility of this process[31]. For example, poly (ADP-ribose) polymerase (PARP) and the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) have been implicated in DNA repair pathways. Proteolytic inactivation of these proteins may be necessary for the efficient degradation of DNA that is observed during apoptosis.

All of these homeostatic proteins appear to be cleaved during apoptosis at a DXXD motif. Several observations suggest that caspase-3 and/or closely related homologs are responsible for these proteolytic events. First, as discussed above, caspase-3 has a near absolute

Table 1 Cellular proteins degraded during apoptosis

	Cleavage sequence, P_4–P_1
Homeostasis/repair	
Poly (ADP-ribose) polymerase (PARP)	DEVD-G
70 kDa U1 small ribonucleoprotein (U1–70 kDa)	DGPD-G
DNA-dependent protein kinases (DNA-PKcs	DEVD-N
SREBP	DEPD-S
Dd-GDI	DELD-S
PKC-δ	DMQD-N
Structure	
Lamin A	VEID-N
Gas-2	SRVD-G
Fodrin	N.A.
NuMa	N.A.
G-actin	LVVD-N
	ELVD-G

N.A. = not available

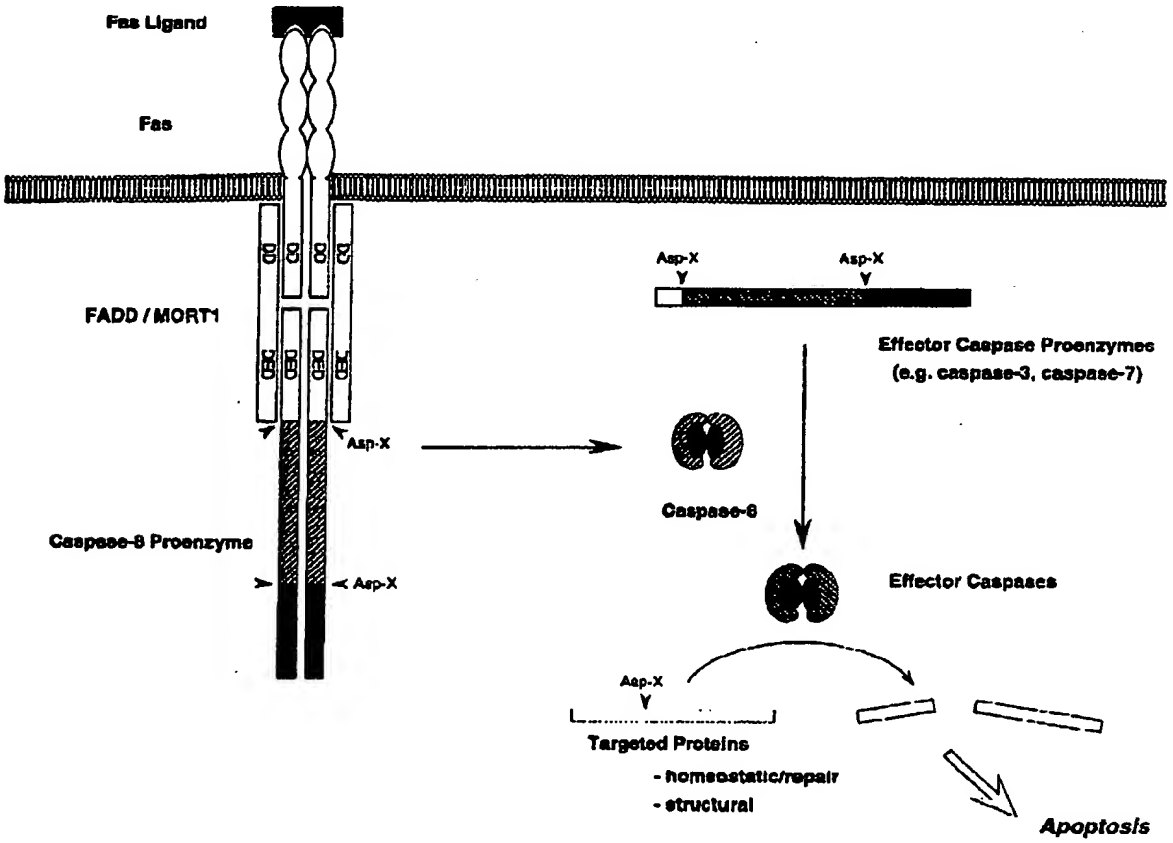

Fig. 2 Proposed model for Fas-induced apoptosis. Excellent evidence for a proteolytic cascade has been obtained in models of Fas-induced apoptosis. It appears that association of Fas ligand to its receptor (Fas/APO-1/CD95) result in formation of a signalling complex that includes the receptor, the adaptor protein FADD/MORT1, and the caspase-8 proenzyme. The interaction between the intracellular domain of Fas and FADD/MORT1 is mediated via dimerization of two homologous regions in the two proteins, termed the death domain (DD). FADD/MORT1, in turn, appears to associate with the proenzyme form of caspase-8 through dimerization of another domain, termed the death effector domain (DED), that occurs in both of these proteins. The subsequent mechanism of activation of caspase-8 is not understood. It is possible that, in the signaling complex, two caspase-8 proenzymes are in close proximity, allowing intermolecular autoproteolysis to occur. The activated protease than goes on to activate effector caspases that function to proteolytically disable proteins essential for maintaining normal cellular functions, and those responsible for the structural integrity of the cell.

requirement for aspartic acid in S_4. Second, PARP, DNA-PK_{cs} and U1–70 kDa subunits of the U1 small ribonucleoprotein (U1–70 kDa) are efficiently cleaved in vitro by caspase-3 with second-order rate constants (k_{cat}/K_m) greater than 1×10^6 $M^{-1}s^{-1}$ [31]. Third, purification of the enzyme responsible for specific PARP cleavage from apoptotic cell cytosols resulted in the isolation of caspase-3[11]. Finally, the inhibition

characteristics of these proteolytic events in extracts from apoptotic cells are consistent with those observed with purified caspase-3 *in vitro*[31]. Evidence that family members other than caspase-3 also have the ability to hydrolyze PARP *in vivo* has been obtained in caspase-3-deficient mice, where cleavage of PARP was observed in thymocytes induced to undergo apoptosis[16].

Structural proteins cleaved during apoptosis include cytoskeletal proteins (fodrin, actin, Gas2), and proteins involved in maintaining nuclear structure (NuMA, lamins). The cleavage of these and other, as yet undefined, proteins may be responsible for many of the observed biochemical and morphologic features that are characteristic of the apoptotic cell, such as surface blebbing and nuclear condensation. The particular caspases responsible for these cleavages have not been firmly established. Unlike the homeostatic proteins described above, in general, the structural proteins do not appear to be cleaved at a DXXD motif, suggesting the involvement of a caspase (or caspases) other than caspase-3. Consistent with this conclusion, lamin A is efficiently cleaved by caspase-6 *in vitro*[15,32].

Regulation of apoptosis by caspases

As noted above, all caspases are synthesized as proenzymes that required proteolytic activation at Asp-X sites to produce the mature, catalytically active heterodimers. It follows that activation is either autocatalytic, or mediated by an enzyme with a similar specificity. The results from several independent investigations support the hypothesis that at least some of these enzymes participate as upstream mediators in a proteolytic cascade that serves to amplify the death signal.

First and most compelling, caspase-8 has been shown to be physically associated with the Fas receptor complex, via an interaction with the adapter protein, FADD/MORT1[33,34]. It appears that this interaction is mediated through dimerization of the death effector domain (DED) of FADD/MORT1, and an analogous domain in the N-terminal peptide of the caspase-8 proenzyme that is removed during activation. Although the mechanism of this activation has not been established, this finding strongly suggests that caspase-8 participates in the signaling activity of the receptor complex during Fas-mediated cell death, and represents the most upstream component in a caspase cascade. It is anticipated that caspase-10, which also contains a DED in its prodomain, will have an analogous function[35]. These findings suggest a rationale for the variable length of the prodomain in caspases. Recently, it has been shown that the prodomain of caspase-1 is absolutely required for its dimerization

and autoactivation[36]. It is tempting to speculate that those caspases with long prodomains have a regulatory role in apoptosis, functioning to activate the caspases with short prodomains which may be unable to dimerize and autoactivate in cells.

Further evidence for a proteolytic cascade comes from investigations into the target(s) of inhibition of apoptosis by both macromolecular and peptide-based inhibitors, which have been shown to prevent activation of those caspases implicated in the effector phase of apoptosis. For example, expression of CrmA in a model of Fas-induced apoptosis prevents activation of both caspase-3 and caspase-7[37]. Similar results are observed with the peptide-based caspase inhibitor, Z-VAD-fluoromethylketone[38]. These results suggest that the target(s) of these inhibitors is a caspase upstream of the effector caspases. Studies of apoptosis induced by Fas ligand suggest this upstream protease has a specificity that is clearly distinct from that of at least some of the downstream enzymes[39].

The serine protease granzyme B has been shown to catalyze the activation of several effector caspases *in vitro*[14,15,35,40–44], leading to speculation that this is one of its primary roles in cytotoxic lymphocyte-mediated cell death. By analogy, it is reasonable to conclude that proteases are also required for the activation of these caspases in other apoptotic systems. Taken together, the findings summarized here strongly support the hypothesis that proteolytic cascades are involved in at least some of the apoptosis that occurs *in vivo*.

Challenges for future research

The frenetic pace of research aimed at understanding the roles of proteases in apoptosis has quickly led to important insights into the biochemical pathways that these enzymes govern. It is clear that these proteases play a central role in most, if not all, of the apoptosis that occurs in mammals. It is also clear that inhibitors of these enzymes, that are likely to be selective for this protease family, prevent the appearance of the morphological features that are characteristic of the apoptotic phenotype, suggesting that these enzymes may be suitable targets for therapeutic intervention. The finding that some of these proteases are involved in the signaling events, in at least some cell types, suggests that intervention can occur before the cell is irreversibly committed to death. The phenotype of caspase-3 deficient mice suggests that this enzyme, in particular, may be an appropriate target for therapeutic intervention in diseases resulting from inappropriate apoptosis in neuronal tissues.

Notwithstanding, several obstacles must be overcome to achieve a therapeutic benefit through inhibition, or activation, of these proteases. First, more studies are required to determine the identities of the caspases involved in mechanisms of cell death in different cell types, and in response to diverse stimuli. In this regard, the phenotype of caspase-3 deficient mice is enlightening, and provocative. Results obtained with these animals strongly suggest that at least some of these enzymes, presumably closely related homologs, have redundant functions. However, it is also clear from these studies that caspase-3 plays a non-redundant role in the apoptosis that occurs during brain development, despite the observation that closely related homologs (caspases 6 and 7) are expressed in neuronal tissues. A better understanding of the key proteases present in different tissues, their functions in these tissues, and their regulation, is required to identify suitable targets for therapeutic modulation.

Second, once an appropriate target in the tissue of interest is identified, selective inhibition or activation of a single caspase may be difficult to achieve. The conclusion that some of these enzymes have redundant functions also implies that their active sites are highly related. Hopefully, structural studies of the various homologs will reveal opportunities for the design of selective inhibitors. Alternatively, there may be significant advances in our ability to deliver drugs to specific tissues. Selective inhibition is expected to be essential for treatment of chronic diseases. In contrast, in cases where premature cell death results from acute injuries (e.g. stroke, ischemia), selective inhibition is probably not as important.

Finally, to attenuate apoptosis *in vivo*, potent, non-peptide inhibitors must be identified that are stable, bioavailable, and able to penetrate cells. The latter is a significant challenge, particularly given that the active sites of these enzymes were designed to accommodate one or more negatively charged entities. In general, charged molecules do not effectively penetrate cells. Consideration of caspase-3 as a potential target for treatment of diseases resulting from excessive neuronal apoptosis presents an even more formidable challenge, as in this case inhibitors will also have to penetrate the blood brain barrier.

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REVIEW ARTICLE

Caspases: the executioners of apoptosis

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Apoptosis is a major form of cell death, characterized initially by a series of stereotypic morphological changes. In the nematode *Caenorhabditis elegans*, the gene *ced-3* encodes a protein required for developmental cell death. Since the recognition that CED-3 has sequence identity with the mammalian cysteine protease interleukin-1β-converting enzyme (ICE), a family of at least 10 related cysteine proteases has been identified. These proteins are characterized by almost absolute specificity for aspartic acid in the P_1 position. All the caspases (ICE-like proteases) contain a conserved QACXG (where X is R, Q or G) pentapeptide active-site motif. Caspases are synthesized as inactive proenzymes comprising an N-terminal peptide (prodomain) together with one large and one small subunit. The crystal structures of both caspase-1 and caspase-3 show that the active enzyme is a heterotetramer, containing two small and two large subunits. Activation of caspases during apoptosis results in the cleavage of critical cellular substrates, including poly(ADP-ribose) polymerase and lamins, so precipitating the dramatic morphological changes of apoptosis. Apoptosis induced by CD95 (Fas/APO-1) and tumour necrosis factor activates caspase-8 (MACH/FLICE/Mch5), which contains an N-terminus with FADD (Fas-associating protein with death domain)-like death effector domains, so providing a direct link between cell death receptors and the caspases. The importance of caspase prodomains in the regulation of apoptosis is further highlighted by the recognition of adapter molecules, such as RAIDD [receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain]/CRADD (caspase and RIP adapter with death domain), which binds to the prodomain of caspase-2 and recruits it to the signalling complex. Cells undergoing apoptosis following triggering of death receptors execute the death programme by activating a hierarchy of caspases, with caspase-8 and possibly caspase-10 being at or near the apex of this apoptotic cascade.

INTRODUCTION

Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and also in chemical-induced cell death [1–3]. Inappropriate apoptosis is implicated in many human diseases, including neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, ischaemic damage, autoimmune disorders and several forms of cancer [4,5]. Apoptosis is a major form of cell death, characterized by a series of distinct morphological and biochemical alterations [1,6]. Apoptotic cell death occurs in two phases: first a commitment to cell death, followed by an execution phase characterized by dramatic stereotypic morphological changes in cell structure [7], suggesting the presence in different cells of a common execution machinery [8]. Apoptosis is characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum (frequently in a subplasmalemmal distribution), a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells, so preventing an inflammatory response [1] (Figure 1). The nuclear alterations, which are the pre-eminent ultrastructural changes of apoptosis, are often associated with internucleosomal cleavage of DNA [9], recognized as a 'DNA ladder' on conventional agarose gel electrophoresis and long considered as a biochemical hallmark of apoptosis. These DNA ladders are derived from large fragments of DNA of 30–50 and 200–300 kbp, which may in terms of higher-order chromatin structure represent loops and rosettes of DNA [10–12]. Internucleosomal cleavage of DNA now appears to be a relatively late event in the apoptotic process, which in some models may be dissociated from early critical steps [13,14]. Nevertheless, its measurement is simple and it is often used as a major criterion to determine whether a cell is apoptotic.

Whereas early studies concentrated on the role of nucleases in apoptosis, more recently a role has been proposed for a number of different proteases, including serine proteases, calpains and proteasomes (reviewed in [15–18]). Most attention has focused on the interleukin-1β (IL-1β)-converting enzyme (ICE)-like proteases, due partly to the enormous progress made by Horvitz and his colleagues in understanding programmed cell death in the nematode *Caenorhabditis elegans*. During normal development, 131 cells of the 1090 cells generated die by apoptosis [2]. Two genes, *ced-3* and *ced-4*, are vital for cell death in *C. elegans*, while the *ced-9* gene antagonizes their function and prevents cell death [19]. The CED-9 protein bears sequence similarity to mammalian Bcl-2, which acts to prevent cell death in mammals. No mammalian homologue of CED-4 has yet been identified. The CED-3 protein bears marked sequence similarity to, and identity with, mammalian ICE [20]. This seminal finding, together with the observation that overexpression of ICE induces apoptosis, suggests that ICE may play a key role in the induction of apoptosis [20,21]. Further evidence supporting a critical role for

Abbreviations used: Ac, acetyl; AMC, 7-amino-4-methylcoumarin; CPP32, 32 kDa cysteine protease; CRADD, caspase and RIP adapter with death domain; DED, death effector domain; DNA-PK, DNA-dependent protein kinase; $DNA\text{-}PK_{CS}$, catalytic subunit of DNA-PK; FADD/MORT1, Fas-associating protein with death domain; ICE, interleukin-1β-converting enzyme; ICH, *Ice* and *ced-3* homologue; $ICH\text{-}1_L$ and $ICH\text{-}1_S$, long and short isoforms respectively of ICH-1; IL-1β, interleukin-1β; PARP, poly(ADP-ribose) polymerase; RAIDD, RIP-associated ICH-1/CED-3-homologous protein with a death domain; Rb, retinoblastoma protein; RIP, receptor-interacting protein; TNF, tumour necrosis factor; TNFR-1, TNF receptor; TRADD, TNFR-1-associated death domain protein; U1-70 kDa, 70 kDa protein component of the U1 small nuclear ribonucleoprotein; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone.

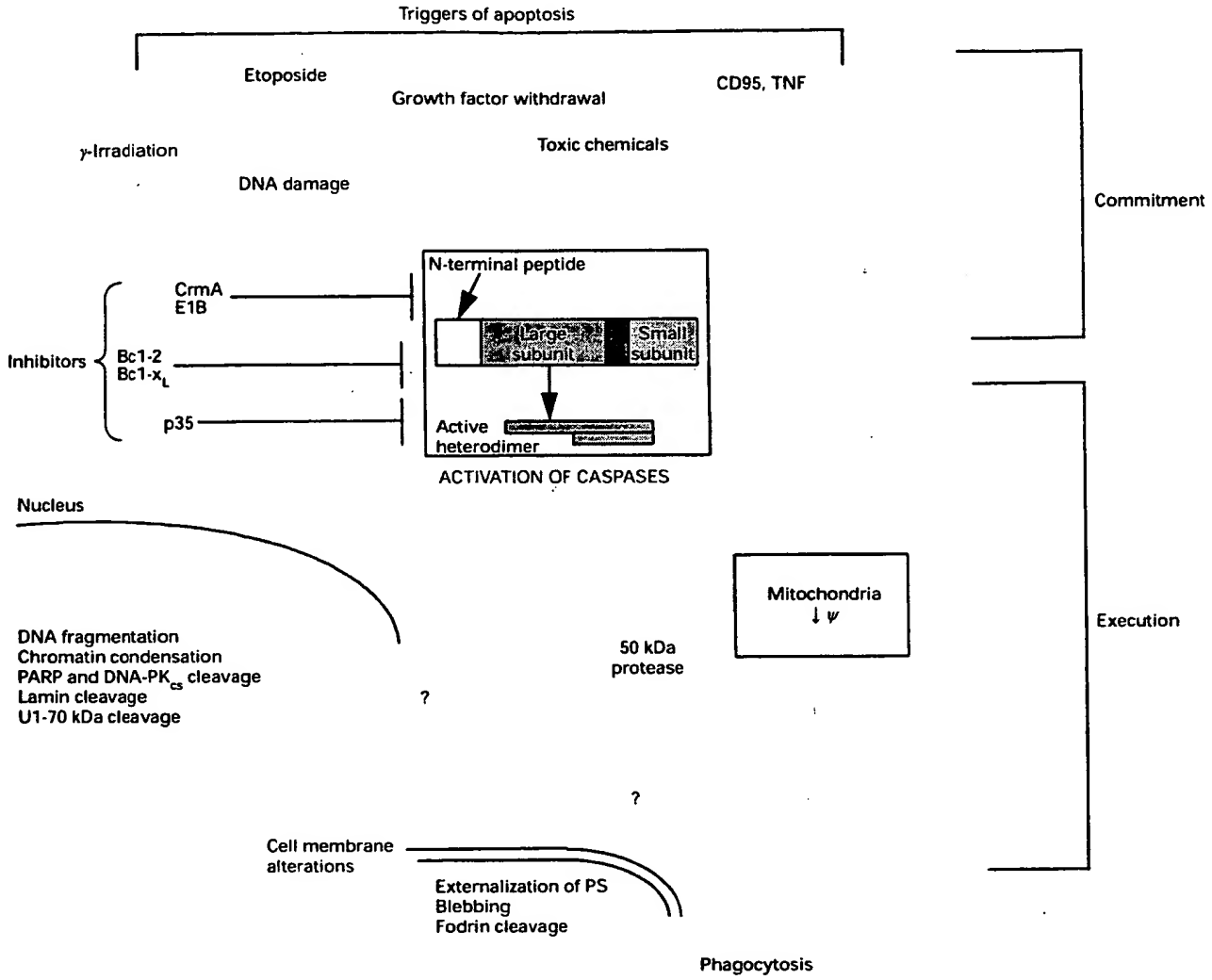


Figure 1 Activation of caspases represents a major control point in apoptosis

Apoptosis involves an initial commitment phase followed by an execution phase. Activation of the caspases results in nuclear, plasma-membrane and mitochondrial changes. The latter include the release of a 50 kDa protease, which may be responsible for some of the cellular changes associated with apoptosis. ψ, mitochondrial membrane potential; PS, phosphatidylserine. See the abbreviations footnote for other definitions.

Table 1 Members of the caspase family

Caspases-3, -7 and -9 have only one cleavage site between the large and small subunits, whereas the other caspases have two potential aspartate cleavage sites, resulting in removal of a linker region. Degrees of inhibition by cowpox viral serpin CrmA: +++, potent inhibition; ±, very weak inhibition; ?, not known.

Caspase	Other names	Active site	Cleavage site(s) between large and small subunits	CrmA inhibition
Caspase-1	ICE	QACRG	WFKD↓S; FEDD↓A	+++
Caspase-2	Nedd2, ICH-1	QACRG	DQQD↓G; EESD↓A	±
Caspase-3	CPP32, Yama, apopain	QACRG	IETD↓S	±
Caspase-4	ICE$_{rel}$II, TX, ICH-2	QACRG	WRVD↓S; LEED↓A	+++
Caspase-5	ICE$_{rel}$III, TY	QACRG	WRVD↓S; LEAD↓S	?
Caspase-6	Mch2	QACRG	DVVD↓N; TEVD↓A	±
Caspase-7	Mch3, ICE-LAP3, CMH-1	QACRG	IQAD↓S	±
Caspase-8	MACH, FLICE, Mch5	QACQG	VETD↓S; LEMD↓L	+++
Caspase-9	ICE-LAP6, Mch6	QACGG	DQLD↓A	?
Caspase-10	Mch4	QACQG	SQTD↓V; IEAD↓A	±

ICE-like proteases in apoptosis is the ability of specific protease inhibitors, including the cowpox viral serpin CrmA [21–23] and baculovirus p35 [24], to inhibit apoptosis (see below).

Since the recognition of the similarity between CED-3 and ICE in 1993, a further nine related ICE-like proteases have been identified. Several reviews have appeared on ICE and ICE-related proteases [7,15,16,25–29]. Due to the flurry of activity to isolate new family members, confusion has arisen as a result of different groups isolating the same protease. In order to resolve this, a unified nomenclature has recently been suggested [30] (Table 1). The trivial name proposed for all family members is caspase, the 'c' denoting a cysteine protease and the 'aspase' referring to the ability of these enzymes to cleave after an aspartic acid residue. Individual family members are then referred to in order of their publication, so ICE, the first family member, is caspase-1. Caspases are synthesized as inactive proenzymes, which are activated following cleavage at specific aspartate cleavage sites. Phylogenetic analysis of the caspases reveals that there are three subfamilies: an ICE subfamily, comprising

caspases-1, -4 and -5, a CED-3/CPP32 (32 kDa cysteine protease) subfamily, comprising caspases-3, -6, -7, -8, -9 and -10, and an ICH-1 (where ICH is *Ice* and *ced-3* homologue)/Nedd2 subfamily (Figure 2a and Table 2).

The presence of a family of structurally related caspases in cells raises a number of important questions in relation to their potential roles in cell death. (1) Are all caspases required for cell death, or are some members more important than others? (2) Do all modes of cell death utilize the same caspases? (3) Are caspases activated in series or in parallel? (4) Is one caspase at the apex of an apoptotic cascade? (5) How are these enzymes regulated in order to prevent their unwanted activation and the subsequent demise of the cell? (6) Are there normal cellular substrates for these enzymes? (7) What are their critical cellular substrates that lead to cell death? (8) Do specific caspases degrade specific proteolytic substrates during the apoptotic process? (9) Are the caspases pre-existing or are they synthesized in response to apoptotic stimuli? (10) Are the caspases tissue-specific? In this review, individual caspases will be discussed, paying particular attention to ICE, as most is known about this enzyme, and a detailed consideration of its properties is extremely helpful in understanding those of other family members. Then some of the known protein substrates of the caspases and their relationship with apoptosis will be reviewed.

ICE (CASPASE-1)

Structure and function

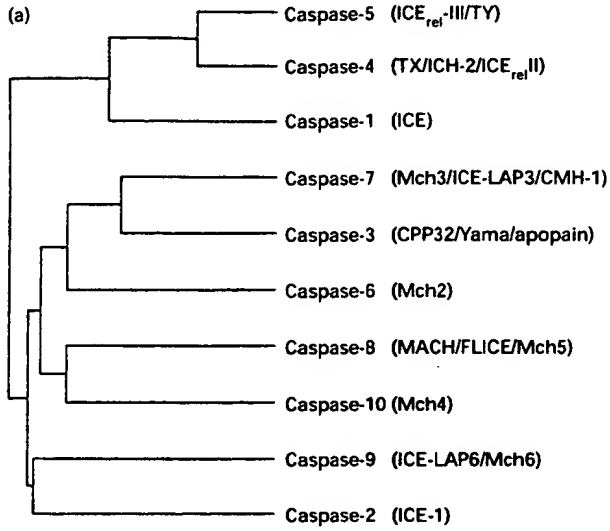
Early work on caspase-1 concentrated on its role in cleaving the inactive 31 kDa cytokine pro-IL-1β at Asp-116–Ala-117 to generate the active 17 kDa mature form of IL-1β, a key mediator of inflammation [31,32]. Purification and cloning of caspase-1 revealed that it is a 45 kDa protein (p45). Active ICE comprises two subunits of 20 kDa and 10 kDa (p20 and p10 respectively), both of which are required for catalytic activity and are derived from a single proenzyme following removal of an 11 kDa N-terminal peptide (prodomain) and a 2 kDa linker peptide (Figure 3) [33,34]. This scheme serves as a useful model for other caspases, although not all possess linker regions and the sizes of the prodomains vary (Figure 3). All four cleavage sites in p45, i.e. Asp-103, Asp-119, Asp-297 and Asp-316, arise at Asp–Xaa bonds (Figure 3), suggesting that active caspase-1 may be derived by autoproteolysis [33,34]. Following the initial cleavage at Asp-297–Ser-298, autoproteolysis occurs in a series of steps, generating fragments of increasing activity and eventually producing p20/p10 ICE [35,36]. Caspase-1 is found predominantly in the cytoplasm of cells as the p45 pro-form [37], although some is also localized to the external cell surface membrane, where it activates pro-IL-1β to its mature form during secretion [38]. Caspase-1 is a novel type of cysteine protease containing an active-site cysteine residue (Cys-285) in the p20 subunit, the mutation of which results in loss of activity. Substrate specificity studies revealed that caspase-1 has a strong preference for aspartic acid adjacent to the cleavage site in the P_1 position, a small hydrophobic amino acid (Gly or Ala) in the P'_1 position, and also a requirement for four amino acids to the left of the cleavage site [33,39]. Acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD.CHO, where CHO is aldehyde) and Ac-YVAD-7-amino-4-methylcoumarin (Ac-YVAD-AMC) were synthesized as a potent competitive reversible inhibitor and a fluorimetric substrate respectively for caspase-1 [33].

The X-ray crystal structure of caspase-1, in complex with specific tetrapeptide inhibitors that bind in the S_1–S_4 sites normally occupied by a peptide substrate, has been determined. The active enzyme is a tetramer of two p20 subunits surrounding two adjacent p10 subunits, with most of the area of contact between the dimers occurring between the p10 subunits [40,41]. Interactions between the p20 C-terminus and the p10 N-terminus also contribute to the stability of the homodimer. The active site spans both the p20 and p10 subunits, so explaining the requirement for both subunits for activity. Cys-285 and His-237 form a catalytic dyad in the active site of caspase-1. The active-site pentapeptide, Gln-Ala-Cys-Arg-Gly (QACRG), is in the p20 subunit. However, amino acid residues involved in forming the Asp pocket include Arg-179, Gln-283, Arg-341 and Ser-347, with only the first two residues being contributed by the p20 subunit. The two arginine residues (Arg-179 and Arg-341) form hydrogen bonds with the P_1 Asp residue of the substrate, and mutation of these residues results in the loss of catalytic activity [41]. Side chains of residues of p10 from Val-338 to Pro-343 interact with P_2–P_4 sites of the inhibitor [41]. Based on the crystal structure, two models were proposed for maturation of the proenzyme. In the first, two precursor p45 proteins associate and are then processed, with the p10 subunit from one caspase-1 molecule complexing with the p20 subunit from another caspase-1 molecule, so creating the active site. The alternative, but less favoured, model suggested processing followed by association of the subunits [41]. Using various active-site mutants, it has been shown that oligomerization of caspase-1 is required for autoprocessing [42] and that hetero-oligomerization may occur between different caspase homologues, but the *in vivo* relevance of this is not known. Using the yeast two-hybrid system, the prodomain of caspase-1 is absolutely required for dimerization and autoproteolysis, suggesting a regulatory rather than a structural role for the prodomain [43]. Using reverse transcriptase–PCR, four alternatively spliced isoforms of caspase-1 were identified that have differing effects on apoptosis [44].

Caspase-1 and apoptosis

Early studies pointed to a role for caspase-1 in the induction of apoptosis. CED-3 and caspase-1 share 28% sequence identity, and the active-site pentapeptide, QACRG, is completely conserved (Table 2) [20]. Overexpression of the murine ICE gene induces apoptosis in Rat-1 fibroblasts, which is abrogated by point mutations in the cysteine or glycine residues of the active-site pentapeptide and by either *bcl-2* or *crmA* [21]. The *crmA* gene (a cytokine response modifier gene) encodes a 38 kDa serpin that is a specific inhibitor of caspase-1, so preventing the proteolytic processing of IL-1β and thereby helping to suppress the response to viral infection [45]. As apoptosis is a major mechanism by which a host attempts to clear virally infected cells, viruses have evolved proteins that inactivate apoptosis in order to infect the host. Dorsal root ganglion neurons, which undergo apoptosis on withdrawal of nerve growth factor, are also protected by *crmA*, suggesting that caspase-1 may be involved in neuronal death in vertebrates [22].

Mice deficient in caspase-1 develop normally, appear healthy and are fertile, with no apparent abnormalities, suggesting that there are no gross defects in normal physiological processes involving apoptosis [46,47]. Macrophages from caspase-$1^{-/-}$ mice are equally susceptible to ATP-induced apoptosis as those from wild-type mice. Thymocytes from caspase-$1^{-/-}$ mice and wild-type mice show a similar susceptibility to apoptosis induced by dexamethasone or γ-irradiation, but thymocytes from the caspase-$1^{-/-}$ mice are somewhat more resistant to apoptosis induced by anti-CD95 (Fas/APO-1) antibody [46,47]. These results suggest that caspase-1 in itself is not involved in most forms of apoptosis, or that another caspase may substitute in the


(a)
Caspase-5 (ICE$_{rel}$-III/TY)
Caspase-4 (TX/ICH-2/ICE$_{rel}$II)
Caspase-1 (ICE)
Caspase-7 (Mch3/ICE-LAP3/CMH-1)
Caspase-3 (CPP32/Yama/apopain)
Caspase-6 (Mch2)
Caspase-8 (MACH/FLICE/Mch5)
Caspase-10 (Mch4)
Caspase-9 (ICE-LAP6/Mch6)
Caspase-2 (ICE-1)

(b)

Large subunit
small subunit
large subunit

Table 2 Sequence identity of the caspases

The sequence identity between the full-length caspases shown in Figure 2(b) was analysed using the GAP program from the Genetics Compter Group.

	Identity (%)										
Caspase	1	2	3	4	5	6	7	8	9	10	CED-3
1	100	22	30	55	50	22	26	22	25	22	29
2		100	22	27	22	28	22	26	33	28	28
3			100	33	30	33	52	33	37	33	34
4				100	77	28	22	20	22	21	26
5					100	22	25	22	24	22	25
6						100	33	35	33	35	35
7							100	33	32	33	33
8								100	22	41	26
9									100	33	29
10										100	25

caspase-1$^{-/-}$ mice. The only exception is the suggestion of a role for caspase-1 in CD95-induced apoptosis in thymocytes. However, no autoimmmune pathologies are seen in the caspase-1$^{-/-}$ mice similar to those caused by the *lpr/lpr* mutation in the murine locus [48].

Further support for a role for caspase-1 in CD95-induced cell death was provided by the findings of a decrease in CD95-induced death due to CrmA, caspase-1 inhibitory peptides and caspase-1-specific antisense oligonucleotides [49,50], together with a small transient increase in a caspase-1-like activity prior to an increase in a CPP32 (caspase-3)-like proteolytic activity [51]. However, this elevation in a caspase-1-like activity has not been demonstrated to be essential for CD95-mediated apoptosis [51]. A role for caspase-1 has also been proposed in the apoptosis of mammary epithelial cells following loss of the extracellular matrix [52] and in DNA-damage-induced interferon regulatory factor-1-dependent T-lymphocyte apoptosis [53]. Similarly, caspase-1 is activated by growth factor deprivation, and suppression of this activation by growth factors such as insulin-like growth factor-1 and insulin also inhibits cell death [54]. In contrast, caspase-1 activity and apoptosis are uncoupled in macrophages undergoing apoptosis [55]. While there is some experimental support for a role for caspase-1 in apoptosis, in particular in CD95-mediated apoptosis, most data (see also below) suggest that other caspases may be of greater significance than caspase-1.

ICH-1/Nedd2 (CASPASE-2)

Nedd2 was originally identified using subtraction cloning as a developmentally down-regulated gene in mouse brain [56,57]. Using a murine *Nedd2* cDNA, a human foetal brain cDNA library was screened at low stringency, and *Ich-1*, the human homologue of *Nedd2*, was identified [58]. Both *Nedd2* and *Ich-1* encode proteins similar to caspase-1, and sequence alignment shows conservation of many important residues, including the active-site pentapeptide QACRG (Table 1 and Figure 2) [57,58]. The *Ich-1* mRNA is alternatively spliced into two forms, one encoding a protein of 435 amino acids (ICH-1_L) and the other encoding a protein of 312 amino acids (ICH-1_S; a truncated form of ICH-1_L) [58]. Overexpression of *Ich-*1_L in some, but not all, cell types results in apoptosis, whereas overexpression of *Ich-*1_S suppresses apoptosis induced by serum withdrawal, suggesting that *Ich-1* may play a role in both the positive and negative regulation of programmed cell death [58]. The enzymic activities of ICH-1_L/Nedd2 are required to cause cell death, as overexpression of mutant *Ich-*1_L*/Nedd2* (with a Ser or Gly respectively replacing the active-site Cys) results in loss of activity [57,58]. During embryonic development, *Nedd2* is expressed at relatively high levels in various tissues, including the central nervous system, liver, kidneys and lungs [57]. Both the kidney and central nervous system are associated with high levels of programmed cell death during development [59]. *Nedd2* is also expressed to varying extents in several adult tissues, including post-mitotic neurons [57].

Two recent studies have addressed the question of which Asp cleavage sites are important in the processing of Nedd2 [60,61]. The Asp-333 → Ala mutant lacks apoptotic activity and does not produce p20 and p10 fragments, suggesting it as the cleavage site at the C-terminus of p20. Asp-347 was identified as the Asp residue at the N-terminus of p10. Asp-135 appears to be a cleavage site upstream of p20, as cell death activity and processing are prevented when it is mutated [60,61]. The proposed cleavage sites in Nedd2 are conserved in caspase-2 (Figure 3). Caspase-2 may be activated *in vitro* by caspase-1, caspase-3 and granzyme B [61]. Recently, the cleavage of caspase-2 to its catalytically active subunits during the execution phase of apoptosis in the human monocytic tumour cell line THP.1 was demonstrated [62]. Caspase-2 was cleaved early during the apoptotic process, but it was not possible to discern whether its cleavage preceded that of other caspases. As yet, no specific intracellular protein substrates of caspase-2 have been identified.

CPP32/YAMA/APOPAIN (CASPASE-3)

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which are cleaved in many different systems during apoptosis (see below). Using the DNA sequence encoding the active site of caspase-1 and CED-3 to search an expressed sequence tag database, a human sequence was identified, cloned and shown to encode a 32 kDa cysteine protease, called CPP32 [63]. Independently, two other groups identified caspase-3, one naming it Yama (the Hindu god of death) and the other apopain [64,65]. Caspase-3, a member of the CED-3 subfamily of caspases (Table 2 and Figure 2b), is widely distributed, with high expression in cell lines of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system [63]. Based on the known cleavage site of PARP (DEVD↓G), Ac-DEVD-AMC was

Figure 2 (a) Phylogenetic relationships and (b) sequence alignment of the caspases

Phylogenetic relationships were determined and polypeptide sequences for the human forms of the caspases aligned using the PILEUP algorithm (Genetics Computer Group, Madison, WI, U.S.A.). The relationships are based on the full-length proenzymes, which is appropriate as the prodomains clearly play a functional role. The dendogram is not affected if the same analysis is carried out using caspases without their prodomains. Amino acid residues are numbered to the right of each sequence. The QACXG motif is conserved in all family members and is boxed. A coloured arrow above the aligned sequences indicates the position of the catalytic cysteine residue. Amino acids that align with residues within ICE that have been shown by the X-ray crysta' structure to be involved in binding P_1 Asp (○), in catalysis (□) and adjacent to substrate P_2–P_4 amino acids (◆) are indicated. The underlined sequences represent the FADD/MORT1 (see the text) homology domains in caspase-8 and caspase-10.

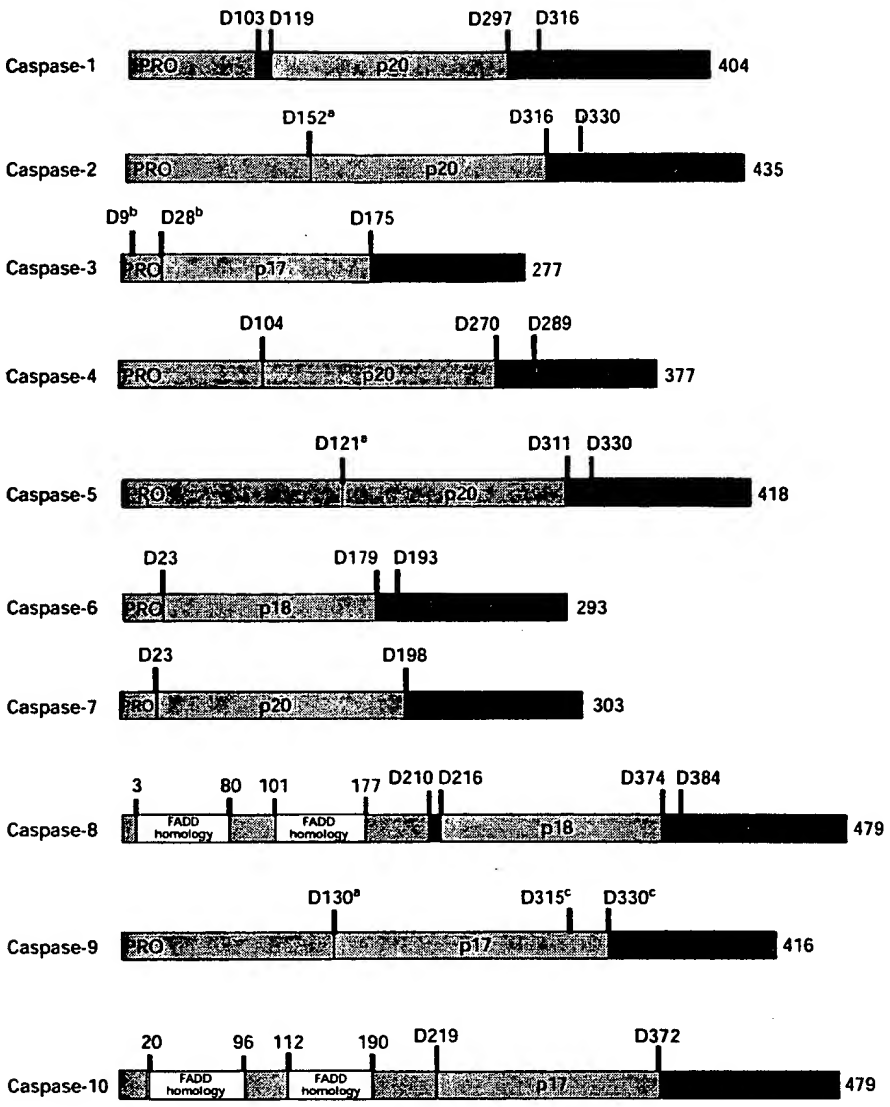


Figure 3 Proenzyme organization of the caspases

Caspases are synthesized as proenzymes, with a N-terminal peptide or prodomain (PRO), and two subunits sometimes separated by a linker peptide (black box). Based on caspase-1 and caspase-3, active enzymes are heterotetramers of two large (~ 20 kDa; p20) and two small (~ 10 kDa; p10) subunits. The proenzymes are cleaved at specific Asp residues (D*n*, where *n* is the position in the protein). The numbers at the right-hand side are the numbers of amino acids in the protein. [a]Exact cleavage site not known; [b]the cleavage site of caspase-3 may be at Asp-9 or Asp-28 [65–67]; [c]caspase-9 is cleaved preferentially at Asp-330 by caspase-3 and at Asp-315 by granzyme B [82]. Caspase-2 cleavage sites are based on equivalent sites being present in Nedd2 [60,61]. FADD represents the domains of caspase-8 and caspase-10 that are homologous to the DED of FADD/MORT1.

synthesized as a model substrate, and Ac-DEVD.CHO and its biotinylated derivatives were synthesized as specific inhibitors of PARP cleavage and as affinity ligands for purification of the protease. Using electrospray MS and N-terminal sequence analysis, the active enzyme was shown to be composed of two subunits of 17 kDa and 12 kDa, derived from the precursor protein by cleavage at Asp-28–Ser-29 and Asp-175–Ser-176 [65] (Figure 3). While the initial cleavage is probably between the large and small subunits, it has been suggested that processing within the prodomain occurs initially at Asp-9, not at Asp-28 [66,67].

During the execution phase of apoptosis, caspase-3 is responsible either wholly or in part for the proteolysis of a large number of substrates, each of which contains a common Asp-Xaa-Xaa-Asp (DXXD) motif (Table 3), similar to that originally described in PARP [68]. In comparison with caspase-1, caspase-3 has no linker peptide and the prodomain is much shorter (Figures 2b and 3). Caspase-3 prefers a DXXD-like substrate, whereas caspase-1 prefers a YVAD-like substrate [65]. Both enzymes have an almost absolute requirement for an Asp in the P_1 position and both can tolerate a fair degree of substitution in the P_2 and P_3 positions, but in the P_4 position caspase-1 prefers a hydrophobic amino acid such as Tyr, whereas caspase-3 has a marked preference for an Asp. The three-dimensional structure of a complex of caspase-3 with DEVD.CHO, a potent tetrapeptide aldehyde inhibitor, shows that, although caspase-3 resembles caspase-1 in overall structure, its S_4 subsite is very different in size and chemical composition and accounts for their differences in specificity [69]. The S_4 subsite of caspase-1 is a large shallow hydrophobic depression that readily accommodates a tyrosyl side chain, while the analogous site in caspase-3 is a narrow pocket that closely surrounds the P_4 Asp side chain [69].

Table 3 Protein substrates of caspases

Abbreviation: SREBP, sterol regulatory element binding protein.

Protein substrate	Cleavage motif	Caspase(s)	Function of substrate	References
PARP	DEVD↓G	3,7	DNA repair enzyme	68, 104
U1-70 kDa	DGPD↓G	3	Splicing of RNA	106
DNA-PK_{cs}	DEVD↓N	3	DNA double-strand-break repair	106, 107
Gas2	SRVD↓G	?	Component of microfilament system	126
Protein kinase C δ	DMQD↓N	3	Cleaved to active form in apoptosis	130, 131
Pro-IL-1β	YVHD↓A	1	Cleaved to mature active cytokine	32–34
D4-GDP dissociation inhibitor	DELD↓S	3	Regulator of Rho GTPases	110
Lamin A	VEID↓N	6	Assist in maintaining nuclear shape	113–116
Heteroribonuclear proteins C1 and C2	?	3,7	Processing of pre-mRNA	108
Huntingtin	DXXD	3	Huntington disease gene product	111
SREBP-1 and SREBP-2	DEPD↓S	3,7	Sterol regulatory element binding proteins	109
Fodrin	DETD↓S?	?	Membrane-associated cytoskeletal protein	127–129
Rb (see the text)	DEAD↓G	3	Cell cycle regulatory protein	112, 133, 134

The Trp residue at position 348 and an inserted 10-amino-acid sequence at position 381 (for ease of comparison, the residue numbers used are those of the analogous residues in caspase-1) play a crucial role in defining the size and shape of the S_4 subsite of caspase-3 and are also conserved in all known members of the CED-3 subfamily (Figure 2b). The activation of caspase-3 to either of its catalytically active p17 or p12 subunits has been demonstrated in cells undergoing apoptosis [70–72].

Caspase-3-deficient mice, generated by homologous recombination, are smaller than their littermates and die at 1–3 weeks of age. Thymocytes from caspase-3-deficient mice show a similar sensitivity to apoptosis induced by a number of different stimuli, including CD95, anti-CD3, staurosporine and dexamethasone. Brain development in these deficient mice is markedly affected, with a variety of hyperplasias being observed from embryonic day 12. Pyknotic clusters of apoptotic cells, observed at sites of major morphogenetic change in normal brain development, are not seen in the deficient mice, indicative of decreased apoptosis in the absence of caspase-3. This demonstrates that caspase-3 plays a critical role during morphogenetic cell death in the mammalian brain and also that mutation of a mammalian homologue of *Ced-3* leads to decreased cell death and a supernumerary cell population during development, emphasizing that the basic cell death machinery is evolutionarily conserved. The restricted phenotype also raises the possibility that other caspases may be important in other tissues or cell types [73].

Cytotoxic T lymphocytes kill target cells containing foreign antigens by either CD95- or granule-mediated cytotoxicity. Exocytosis of cytotoxic T lymphocyte granules allows perforin to polymerize in the target cell membrane, so facilitating entry of the granzymes, a family of serine proteases. Granzyme B also exhibits an unusual specificity for Asp in the P_1 position. *In vitro*, granzyme B can activate caspase-3, as assessed by formation of its p17 subunit as well as its ability to cleave PARP to its signature fragment [74,75]. Activation of caspase-3 by granzyme B is within the physiological range [75]. While granzyme B can activate directly caspase-3, it may also cleave another caspase which in turn activates caspase-3.

ICE_{rel}II/TX/ICH-2 (CASPASE-4) AND ICE_{rel}III/TY (CASPASE-5)

Three groups independently cloned ICE_{rel}II/TX/ICH-2 (caspase-4) [76–78] and two groups cloned ICE_{rel}III/TY (caspase-5) [76,79] (Figure 2b). Both caspase-4 and caspase-5 are members of the caspase-1 subfamily, and are more closely related to each other than to other homologues (Table 2). Caspase-4 expression, while lower than that of caspase-1, generally shows a similar tissue distribution, being found in most tissues examined with the exception of brain. Appreciable levels are found in both lung and liver, and also in ovary and placenta, where caspase-1 mRNA is barely detectable [76,77]. Caspase-5 is expressed at a much lower level than caspase-4 [76,79]. Caspase-4 and caspase-5 have different substrate specificities from that of caspase-1, being much poorer at cleaving pro-IL-1β [76–79]. Caspase-4 may be involved in the maturation of caspase-1 [78]. At high concentrations, caspase-4 cleaves PARP [80], but the biological relevance of this is unclear. Limited information is available concerning the precise sites at which caspase-4 and caspase-5 are processed. Maturation of caspase-1 results from cleavage after Asp residues at positions 119, 297 and 316, with the latter two residues being conserved in caspase-4 (Asp-270 and Asp-289) and caspase-5 (Asp-311 and Asp-330); by analogy, this probably represents the removal of a linker peptide (Table 1 and Figure 3). The Asp-119 site in caspase-1 is not conserved in caspase-4, but Asp-104 might define the cleavage site for removal of the prodomain [77]. Overexpression of caspase-4 and caspase-5 generally results in apoptosis, although in some studies apoptosis was only induced after removal of the prodomains, suggesting that the prodomains may be involved in the regulation of apoptosis [76,78].

Mch2 (CASPASE-6)

Using a PCR approach with degenerate primers encoding two highly conserved pentapeptides, QACRG and GSWFI, Alnemri and co-workers cloned Mch2 (a mammalian CED-3 homologue) from a human Jurkat T lymphocyte cDNA library [81]. Two transcripts were detected, Mch2α (1.7 kb) and Mch2β (1.4 kb), the former encoding the full-length Mch2 and the latter encoding a shorter isoform, possibly as a result of alternative splicing. Mch2α (caspase-6) encodes a 293-amino-acid protein with a predicted molecular mass of $\sim$34 kDa. Mch2β contains a deletion corresponding to nucleotides 119–385 of the Mch2α sequence (amino acids 14–102) and encodes a 204-amino-acid protein with a predicted molecular mass of $\sim$23 kDa. Mch2β lacks half of the p20 subunit and is probably catalytically inactive [81]. Expression of Mch2α, but not Mch2β, in insect cells results in the induction of apoptosis. Caspase-6 is a member of

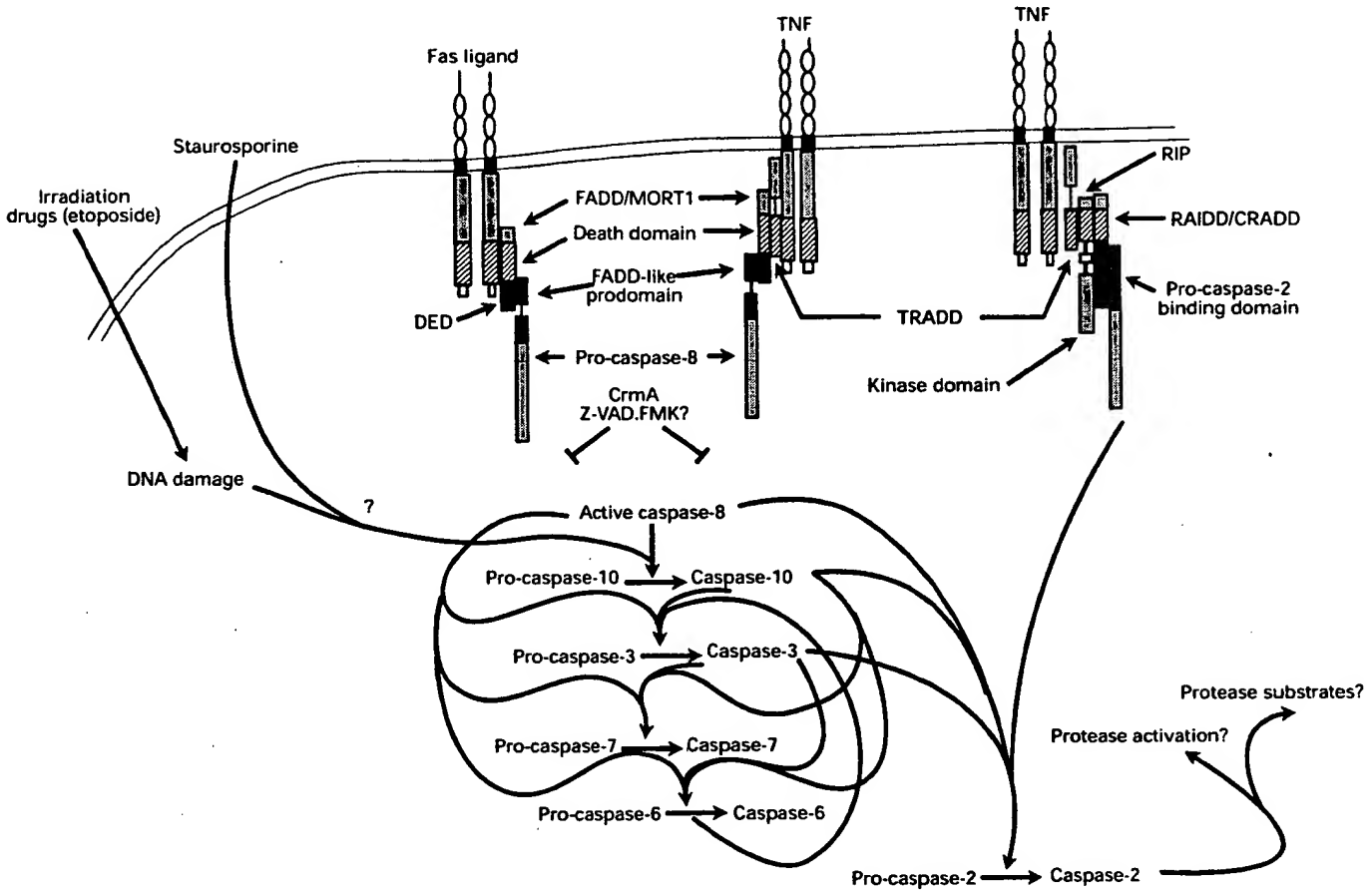


Figure 4 Hypothetical hierarchy of caspases

Apoptosis can be triggered by drugs (staurosporine), by DNA-damaging agents (etoposide or γ-irradiation) or by CD95 (Fas/APO-1) or tumour necrosis factor (TNF) interacting with their respective receptors. Both of these receptors transduce their apoptotic signals via intracellular C-terminal death domains (hatched boxes) which are involved in protein–protein interactions. Both CD95 and TNF result in the cleavage and activation of caspase-8, which can cleave all other known caspases. Caspase-10, which also cleaves all other known caspases, is activated by different stimuli via a CrmA-insensitive pathway, and may be activated following DNA damage (?). Activation of some caspases, such as caspase-6, results in their being able to cleave the caspase initially responsible for their formation, so setting up a protease amplification cycle [82]. Caspases may also be recruited to the death pathways by adapter molecules such as RAIDD/CRADD, which bind to RIP and to the prodomains of certain caspases such as pro-caspase-2 [101,102]. Part of this scheme, showing which caspase activates other caspases, is based on *in vitro* studies with purified enzymes, and requires confirmation from studies with intact cellular systems.

the CED-3 subfamily, showing high identity with caspase-3 (Figure 2a and Table 2).

Caspase-6, when expressed as a glutathione S-transferase fusion protein in *Escherichia coli*, autoprocesses and cleaves Ac-DEVD-AMC [although it is much less active ($\sim$ 150-fold) than caspase-3], but it does not cleave Ac-YVAD-AMC. Caspase-6 is also not readily inhibitable by Ac-DEVD.CHO [82]. Recombinant caspase-6 cleaves human PARP to give a smaller fragment than that seen in cells undergoing apoptosis, suggesting that caspase-6 does not play a major role in the cleavage of PARP [81]. Purified human recombinant caspase-3 cleaves ^{35}S-labelled pro-caspase-6 at three aspartate cleavage sites (Asp-23, Asp-179 and Asp-193), resulting in the formation of the large (p18) and small (p11) subunits of caspase-6 [82]. Initial cleavage appears to be at $D^{176}VVD\downarrow N^{180}$, resulting in a p13 subunit, which is then further cleaved at $T^{190}EVD\downarrow A^{194}$, resulting in the p11 subunit (Figure 3). Activation of pro-caspase-6 by caspase-3 results in an active enzyme that is capable of cleaving an artificially introduced lamin cleavage site (VEID$\downarrow$N) [82]. These data suggest that caspase-3 is activated prior to, and may be responsible for, the activation of caspase-6. However, these results are in apparent conflict with a recent study which identified and purified from hamster liver a homologue of caspase-6 that is capable of activating caspase-3 [83]. In addition, caspase-6 processes pro-caspase-3 at the $I^{172}ETD\downarrow S^{176}$ site between the large and small subunits, and this cleavage is blocked when Asp-175 is mutated to Ala [82]. Thus activation of caspase-3 can result in activation of pro-caspase-6 but, similarly, activation of pro-caspase-6 can also result in activation of caspase-3, resulting in a protease amplification cycle (Figure 4) [82]. Some caution must be exercised in the interpretation of such *in vitro* experiments, as purified or partially purified proteases may cleave substrates that they would not cleave *in vivo*, either because the ratio of enzyme to substrate would never reach such a level in the cell or because the enzyme may be located in a different subcellular environment from the substrate.

Mch3/ICE-LAP3/CMH-1 (CASPASE-7)

Caspase-7 was cloned independently in three different laboratories and named Mch3/ICE-LAP3/CMH-1 [84–86]. A member of the CED-3 subfamily, it is a 303-amino-acid protein with high

similarity to caspase-3 (Figure 2a and Table 2). An alternatively spliced isoform of caspase-7, which may act as a negative regulator of apoptosis, has been described [84]. Caspase-7 is constitutively expressed in many foetal and adult tissues, with lowest expression observed in the brain. Using a rabbit anti-peptide antibody, caspase-7 protein migrates at ~ 35 kDa and is detected to a variable extent in a number of cell lines, including Jurkat T cells, where it is localized diffusely to the cytoplasm and juxtamembrane structures [85], consistent with the suggestion that the death effector machinery resides in the cytoplasm rather than the nucleus [8]. Overexpression of full-length caspase-7 in the MCF7 breast carcinoma cell line does not induce apoptosis, whereas expression of a truncated derivative, lacking the 53 N-terminal amino acids corresponding to the putative prodomain, induces apoptotic cell death [85]. Bacterially expressed caspase-7, like caspase-3, preferentially cleaves PARP and the peptide substrate Ac-DEVD-AMC, but not Ac-YVAD-AMC or pro-IL-1β. The competitive peptide aldehyde inhibitor Ac-DEVD.CHO is a potent inhibitor of both caspase-3 and caspase-7, whereas Ac-YVAD.CHO and CrmA are poor inhibitors of both of these enzymes. As caspase-3 and caspase-7 are functionally similar and have similar substrate specificities [84], cleavage of PARP during apoptosis may be due to a combination of the action of both these caspases. Active caspase-7 is made up of two subunits, similar to other caspases [66] (Figure 3). Caspase-7 is activated to its catalytically active large subunit in intact cells undergoing apoptosis [62,85,87].

Following cleavage at Asp-198 and Asp-23, granzyme B activates pro-caspase-7 to a form that cleaves PARP to its signature fragment of ~ 85 kDa [66,88,89]. Using a caspase-7 protein with Asp-23 mutated to glutamate, it was demonstrated that removal of the prodomain by autoprocessing is not necessary for activity and that the p25/p12 enzyme is as active as the p20/p12 enzyme [89]. This suggested that the prodomain of caspase-7 may be involved in an as yet uncharacterized way in the regulation of caspase-7. *In vitro*, caspase-7 is a better substrate for granzyme B than is caspase-3. Pro-caspase-7 appears to exist as dimers or higher-order oligomers [89]. Incubation of Jurkat T cells with granzyme B together with a sublytic concentration of perforin results in the activation of endogenous caspase-7 prior to the induction of apoptosis. Together, these data suggest that caspase-7 may be an important intracellular effector of granzyme B-mediated apoptosis and cytotoxic T-lymphocyte-induced cell killing *in vivo*.

MACH/FLICE/Mch5 (CASPASE-8)

CD95 and the TNF receptor (TNFR-1; p55-R) are members of the TNF/nerve growth factor receptor family. Activation of these cell-surface cytokine receptors, either by their natural ligands or by agonistic antibodies, results in apoptosis. CD95 and TNFR-1 share a region of identity termed the 'death domain' that is required to signal apoptosis. Using a yeast two-hybrid system, three proteins were identified that bind to either the intracellular domains of CD95 and/or TNFR-1 through hetero-association of homologous regions found in these proteins. FADD/MORT1 (Fas-associating protein with death domain) [90,91] binds specifically to CD95, TRADD (TNFR-1-associated death domain protein) binds to TNFR-1 [92], and RIP (receptor-interacting protein) binds to both receptors [93]. Activation of CD95 initiates the association of at least four proteins (CAP1–CAP4), two of which (CAP1 and CAP2) have been identified as alternative forms of phosphorylated FADD/MORT1 [94]. A dominant-negative version of FADD, lacking the N-terminal death effector domain (DED), blocks the recruitment of the two other proteins (CAP3 and CAP4) to the death-inducing signalling complex [91], suggesting that these two proteins are downstream components of the CD95 signalling cascade. The region encompassing the 117 N-terminal amino acid residues of FADD can trigger apoptosis, and have been called DED [95].

Two groups independently identified a novel caspase, named MACH/FLICE (caspase-8), which contains both an active subunit with identity with the caspases and an N-terminal prodomain containing two domains with marked identity with the N-terminal DED of FADD/MORT1 [96,97] (Figure 3). Using nano-electrospray tandem MS, peptide sequences were obtained for CAP4 which, when used as sequence tags, identified a 3 kb cDNA that encodes a novel protein, caspase-8, of predicted molecular mass 55.3 kDa. The other group used a yeast two-hybrid screen to identify a cDNA clone with a novel sequence which binds to MORT1/FADD. This novel protein (MACH) occurs in multiple isoforms, most probably produced by alternative splicing, some of which contain a region with identity with the caspases. Northern blot analysis revealed a heterogeneity of caspase-8 transcripts which varied in amount and size in different human tissues. Few caspase-8 transcripts are detectable in testis, skeletal muscle and brain, with a relatively higher level of expression in peripheral blood leucocytes, consistent with a role for CD95-induced apoptosis in lymphocyte homoeostasis.

Caspase-8 contains two N-terminal stretches of approx. 70 amino acids that are apparently homologous to the DED of FADD. Residues 7–75 and 101–169 of caspase-8 share 39% identity (55% similarity) and 28% identity (55% similarity) respectively with the DED of FADD (residues 4–76) [97]. While the N-terminal portion of caspase-8 contains the FADD homology domains, the remainder of the protein is highly similar to the CED-3 subfamily of caspases (Figure 2a and Table 2). Instead of the active-site QACRG pentapeptide found in most caspases, caspase-8 contains the novel sequence QACQG. Caspase-8 associates with the DED of FADD. Granzyme B activates caspase-8 to an active protease which cleaves PARP to its characteristic signature fragment. Overexpression of caspase-8 results in apoptosis, and mutation of its catalytic cysteine residue abolishes its apoptotic potential. Expression of caspase-8 in the presence of isoforms with an incomplete caspase region resulted in little cell death. Similarly, these isoforms lacking a complete caspase region blocked cell death induced by CD95 and TNF, suggesting that the isoforms exert a dominant-negative effect and may be important in the regulation of apoptosis *in vivo*.

The marked heterogeneity of isoforms of caspase-8 compared with other caspases may provide a mechanism for some tissues or cells to protect themselves against CD95- or TNF-induced cell death [96]. These studies provide a critical link between activators and effectors of the cell death machinery. Oligomerization of the death domain of either CD95 or TNFR-1 allows recruitment of cytosolic adapter proteins to assemble a death-inducing signalling complex [98]. Thus CD95 (a cell death receptor) uses FADD (an adapter molecule) to interact physically with caspase-8 (a cytosolic protease) and initiate the apoptotic cascade (Figure 4). The precise mechanism by which recruitment of caspase-8 results in its activation is not known. It has been suggested that, in the latent state, the two DEDs of caspase-8 bind to each other, so preventing activation. The binding of FADD following the triggering of apoptosis by CD95 or TNF may cause a conformational change in the DED of FADD, so facilitating its binding to one of the DEDs of caspase-8, thereby disrupting the association of the two DEDs of caspase-8 and allowing its caspase domain to undergo autocatalytic activation [96,97].

Independently, Mch5 was cloned from Jurkat T cells, and its predicted sequence is almost identical with that of caspase-8 [66,96]. A small difference in the prodomain is revealed following sequence comparison of Mch5 and MACH/FLICE (G. M. Cohen, unpublished work). Bacterial expression of pro-caspase-8 generates a mature enzyme composed of two subunits, derived by processing of the proenzyme (Figure 3). Recombinant caspase-8 is able to process/activate all known caspases, including caspases-1 to -7 and caspases-9 and -10 [66,67], supporting the suggestion that it lies at the apex of an apoptotic cascade, at least from some stimuli such as CD95 or TNF [96,97].

ICE-LAP6/Mch6 (CASPASE-9)

Recently, two groups independently cloned a new member of the caspase family, ICE-LAP6/Mch6 (caspase-9) [82,99]. On searching the databases for genes related to that for caspase-7, a cDNA clone was identified that encodes a novel 416-amino-acid protein with a predicted molecular mass of ~ 46 kDa. Caspase-9 is a member of the CED-3 subfamily, bearing high similarity to caspase-3 (Table 2). The major difference between caspase-9 and other family members is the active-site pentapeptide QACGG, in which a Gly is found instead of the usual Arg (Table 1). Pro-caspase-9 contains a long N-terminal putative prodomain with high similarity to the prodomains of CED-3 and caspase-2. Northern-blot analysis revealed the presence of multiple mRNA species, suggestive of alternative splicing. High levels of expression of caspase-9 are found in the heart, testis and ovary. Overexpression of caspase-9, but not of a mutant in which the catalytic Cys was replaced with an Ala, induced apoptosis in MCF7 cells.

Procaspase-9 contains two potential processing sites between its large and small subunits, $P^{312}EPD\downarrow A^{316}$ and $D^{327}QLD\downarrow A^{331}$. The latter motif is similar to the DEVD↓G site in PARP, suggesting that caspase-9 may be activated by caspase-3, while the former motif may be a potential granzyme B cleavage site, as it contains an acidic residue in the P_3 position. Using *in vitro* mutagenesis, it was demonstrated that both caspase-3 and granzyme B activated pro-caspase-9, although to differently sized products. Asp-330 was the processing site for caspase-3, generating two products of molecular masses ~ 37 kDa (p37) and ~ 10 kDa (p10). Granzyme B cleaved procaspase-9 at both sites, with a marked preference for Asp-315 over Asp-330, generating an active enzyme capable of cleaving PARP to its signature fragment of ~ 85 kDa. In some, but not all, studies the prodomain of caspase-9 was removed [82,99]. The ability of caspase-3 to activate pro-caspase-9 suggests that the latter is downstream of caspase-3 and, as such, may be responsible for some of the later changes seen in cells undergoing apoptosis.

Mch4 (CASPASE-10)

Searching the databases of expressed sequence tags for sequences related to caspase-3 and caspase-6 led to the cloning from Jurkat T cells of a novel cDNA encoding a 479-amino-acid protein, Mch4 (caspase-10), with a molecular mass of ~ 55 kDa [66]. Caspase-10, a member of the CED-3 subfamily, is more closely related to caspase-8 than to any other caspase (Figure 2a and Table 2). Like caspase-8, caspase-10 has an active-site QACQG pentapeptide and also contains two FADD-like DEDs in its N-terminal domain, suggesting a possible role in CD95- or TNF-induced apoptosis. Mature caspase-10 is derived from a single-chain polypeptide proenzyme by cleavage at Asp-372 located between the large and small subunits (Figure 3). Northern blot analysis revealed that caspase-10 mRNA is present in most tissues, with lowest expression being observed in brain, kidney, prostate, testis and colon and higher levels in heart, liver and spleen. Recombinant caspase-10 is unusual in that it has a similar K_m for the cleavage of both Ac-DEVD-AMC and Ac-YVAD-AMC, but it is more similar to caspase-3 as it is potently inhibited by Ac-DEVD.CHO [66]. Granzyme B cleaves procaspase-10, lacking the N-terminal FADD-like domains, at $I^{369}EAD\downarrow A^{373}$. Purified recombinant caspase-10 processes all caspases, including pro-caspases-3, -7 and -10, whereas neither caspase-3 nor caspase-7 cleaves pro-caspase-10, suggesting that the latter is upstream of both caspase-3 and caspase-7 and lies at or near the apex of a cascade of proteases [66,67] (Figure 4).

GENERAL FEATURES OF THE CASPASES

Caspases are synthesized as inactive proenzymes which are activated by cleavage at specific Asp residues to active enzymes containing both large (p20) and small (p10) subunits. In some cases these subunits are separated by a linker region of unknown function but which may be involved in regulation of the activation of the caspase. All caspases contain an active-site pentapeptide of general structure QACXG (where X is R, Q or G). The amino acids Cys-285 and His-237 involved in catalysis, and those involved in forming the P_1 carboxylate binding pocket in caspase-1 (Arg-179, Gln-283, Arg-341 and Ser-347), are conserved in all the other caspases, except for the conservative substitution of Thr for Ser-347 in caspase-8 (Figure 2b). This explains the absolute requirement for an Asp in the P_1 position. However, the residues that form the P_2–P_4 binding pocket are not well conserved, suggesting that they may determine the substrate specificities of the different caspases. It is evident from studies such as those with the caspase-$1^{-/-}$ and caspase-$3^{-/-}$ mice that not all caspases are required for cell death, and that some are more important than others. The importance of the tissue specificity of individual caspases is illustrated by the effects on the brains of caspase-$3^{-/-}$ mice [73]. Alternatively spliced isoforms of many caspases may in part regulate the activity of the full-length enzyme, either by acting as dominant inhibitors or by forming inactive heteromeric complexes [58,96]. A key role for a particular caspase in apoptosis has often been inferred from its overexpression resulting in the induction of this process. However, such overexpression may lead to the caspase cleaving substrates that it does not normally recognize. In addition, injection of other proteases such as trypsin or chymotrypsin into the cytoplasm of various cell types results in the induction of apoptosis [100].

Some caspases contain only a short prodomain (caspases-3, -6 and -7), whereas others contain long prodomains (caspases-1, -2, -4, -5, -8, -9 and -10) (Figure 3). The importance of the FADD-like prodomains of caspase-8 and possibly caspase-10 in directly linking CD95- and TNFR-1-mediated apoptosis has already been emphasized [96,97]. The significance of the other prodomains is not known, but they may be important in regulation of the activation of the caspases [43]. This possibility has been highlighted by the identification of a new adapter molecule, RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain)/CRADD (caspase and RIP adapter with death domain) [101,102]. RAIDD/CRADD comprises two main domains: an N-terminal domain which resembles and binds by a homophilic mechanism to the prodomain of caspase-2 and CED-3, and a C-terminal death domain that binds to RIP, a Ser/Thr kinase, which associates with CD95 and induces death [101]. The N-terminus of RAIDD/CRADD has significant identity with the prodomains of caspase-2 (residues 15–91; 31% identity), caspase-9 (residues 1–79; 28% identity) and CED-3

(residues 2–78; 24% identity), as well as with the C-terminus of a human inhibitor of apoptosis (IAP-1) [102]. Mutation of highly conserved residues in the N-terminal prodomains of caspase-2 and CED-3 abolishes their binding to RAIDD. RAIDD binds RIP, part of the TNFR-1 signalling complex, but not FADD or TRADD, except in the presence of RIP, when it can bind the latter. RIP recruits RAIDD/CRADD, which in turn recruits caspase-2, so creating a direct link to the effector caspases [101,102] (Figure 4). Interestingly, mature caspase-2 processes its own precursor, but not other currently known caspases [102]. Thus prodomains of some caspases may enable them to be recruited specifically to facilitate the execution of the cell death programme. It remains to be determined whether there is a family of RAIDD-like molecules that can recruit other caspases with long prodomains.

PROTEIN SUBSTRATES CLEAVED BY CASPASES DURING THE EXECUTION PHASE OF APOPTOSIS

During the execution phase of apoptosis, several proteins, including PARP, lamin B and histone H1, are cleaved [103]. The number of proteins identified as being cleaved during apoptosis is increasing rapidly. The caspases responsible for these reactions are indicated in Table 3; however, other caspases may also cleave these substrates. It is also not known which caspase(s) is/are responsible for cleavage under physiological conditions, or whether there is redundancy in the proteases for these cleavages. Some caspases show overlapping specificities for some substrates (caspase-3 and caspase-7 can both cleave PARP), whereas other caspases may have a unique substrate specificity (to date, caspase-6 is the only caspase known to cleave lamins). The biological significance of these proteolytic cleavages and their relationship with the ensuing apoptotic morphology is often not known. Caspase-3 is responsible, either wholly or in part, for the proteolytic cleavage of a large number of substrates during apoptosis, including PARP, DNA-dependent protein kinase (DNA-PK), U1-70 kDa, heteronuclear ribonucleoproteins C1 and C2, sterol regulatory binding proteins, D4-GDP dissociation inhibitor, huntingtin, and almost certainly retinoblastoma protein (Rb) [104–111] (Table 3). A common feature of all these substrates is the presence of a DXXD motif (Table 3), similar to that originally described in PARP [68]. Based on the cleavage site of PARP, Ac-DEVD-AMC was designed as a fluorimetric substrate for the measurement of caspase-3 activity. However, since caspase-7 also cleaves this substrate and this reaction is also potently inhibited by Ac-DEVD.CHO, it is likely that many substrates described as being cleaved by caspase-3 will also be found to be cleaved by caspase-7.

PARP

PARP is possibly the best characterized proteolytic substrate of caspases, being cleaved in the execution phase of apoptosis in many systems, including thymocytes, HL-60 cells and breast cancer cell lines [103,104]. Intact PARP (116 kDa) is cleaved to 24 kDa and 89 kDa fragments, representing the N-terminal DNA binding domain and the C-terminal catalytic domain of the enzyme respectively [104]. This possibly conserves the cellular NAD^+ and ATP normally required for PARP activity, thereby enabling the ATP to be utilized for the execution of apoptosis [7]. Cleavage of PARP may also interfere with its key homoeostatic function as a DNA repair enzyme [105]. PARP is cleaved at the sequence DEVD↓G by a protease activity resembling ICE (prICE), but not by ICE itself [68]. Although the cleavage of PARP is often a valuable indicator of apoptosis, its biological relevance, if any, is unclear, since PARP-null mice develop normally [112]. *In vitro*, other caspases, including caspases-2, -4, -6, -7, -8, -9 and -10, when added at high concentrations, can also cleave PARP or DEVD-AMC [80]. The physiological significance of this cleavage of PARP by these caspases is still under investigation. At the present time, it appears that caspase-3 and caspase-7 are primarily responsible for PARP cleavage during apoptosis.

DNA-PK

DNA-PK, an enzyme involved in DNA double-strand-break repair, possesses a 460 kDa catalytic subunit ($DNA\text{-}PK_{cs}$) and a DNA binding component Ku, which is a heterodimer of 70 and 80 kDa subunits. During apoptosis in several different systems, $DNA\text{-}PK_{cs}$, but not Ku, is degraded by a caspase with properties similar to those of caspase-3 [105,106]. Caspase-3, but not caspases-1, -4 or -6, cleaves purified $DNA\text{-}PK_{cs}$ to fragments of a similar size to those observed in cells undergoing apoptosis. Degradation of $DNA\text{-}PK_{cs}$ should lead to a decrease in the DNA repair capacity of the cell, so abolishing its key homoeostatic function and facilitating the characteristic DNA degradation associated with apoptosis [105,106].

Lamins

The proteolysis of lamins, the major structural proteins of the nuclear envelope, is observed in different cells undergoing apoptosis [68,87,113–116] and may be responsible for some of the observed nuclear changes, since inhibitors of lamin cleavage prevent some of these changes [115,117]. An *in vitro* model of apoptosis has been developed in which normal nuclei, exposed to cytosol from apoptotic cells, undergo many of the characteristic biochemical and morphological changes of nuclear apoptosis, including chromatin condensation, fragmentation and margination, internucleosomal cleavage of DNA, and proteolysis of PARP and lamins [68,118]. In this model, the lamin protease is clearly distinct from the PARP protease, cleavage of PARP being significantly more rapid than that of lamins and less sensitive to inhibition by either YVAD chloromethyl ketone or tosyl-lysylchloromethane [115]. Caspase-6 (Mch2) cleaves lamin A at a conserved VEID↓N sequence to give a fragment similar to that seen in apoptotic cells and extracts [119,120]. The site of cleavage is in a well conserved α-helical rod domain, which may disrupt lamin–lamin interactions as well as interactions of lamins with other nuclear components [120]. This lamin cleavage is readily inhibited by YVAD chloromethyl ketone. Under conditions where caspase-6 cleaves lamin A, caspases-1, -3 and -7 do not, suggesting that caspase-6 may be the major laminase in cells undergoing apoptosis [119,120]. Other, as yet untested, caspases may also cleave lamins, as may a Ca^{2+}-regulated serine protease [115,121]. Pro-caspase-6 is activated in cells undergoing apoptosis induced either by anti-CD95 antibody or by staurosporine, and caspase-6 functions downstream of Bcl-2 and $Bcl\text{-}x_L$ [119]. In CD95-treated HeLa cells, lamin B is preferentially cleaved early in apoptosis prior to cleavage of lamins A and C and internucleosomal cleavage of DNA; this suggests that B- and A-type lamins may be cleaved by different caspases [122].

U1-70 kDa

The activity of the U1 small nuclear ribonucleoprotein particle, which is essential for the splicing of precursor mRNA, is dependent on both the RNA and protein components, including U1-70 kDa. In several systems, including CD95- and TNFR-

induced apoptosis, U1-70 kDa is cleaved early in the apoptotic process to a 40 kDa fragment [105,123]. Purified caspase-3 cleaves U1-70 kDa at a DGPD↓G site, similar to the reaction observed in apoptotic cells. In lysates from apoptotic cells, the cleavage of U1-70 kDa is potently inhibited by Ac-DEVD.CHO but not by Ac-YVAD.CHO, strongly suggesting that caspase-3 or possibly caspase-7 is the major caspase responsible for its cleavage in apoptosis. Cleavage of U1-70 kDa separates the RNA binding domain from the distal arginine-rich region of the molecule, which may have a dominant-negative effect on splicing; such inhibition of splicing would block cellular repair pathways dependent on new mRNA synthesis [105].

Fodrin

During the execution phase of apoptosis, a number of important plasma-membrane changes occur, resulting in the recognition and subsequent phagocytosis of the apoptotic cell either by a professional phagocyte or by a neighbouring cell. Cleavage of important cytoskeletal proteins, including actin [124,125], Gas2 [126] and fodrin (non-erythroid spectrin) [127–129], during apoptosis may induce cell shrinkage and membrane blebbing, and alter cell survival signalling systems. α-Fodrin, an abundant membrane-associated cytoskeletal protein, is rapidly and specifically cleaved during CD95- and TNF-induced apoptosis, and this appears to be related to the membrane blebbing. Initially it was proposed that fodrin is cleaved by calpain I [127], but the cleavage is probably due to a caspase [128,129]. Fodrin contains several potential caspase cleavage sites, including a DETD↓S site only nine amino acids away from the proposed calpain cleavage site, which may have led to the initial confusion [128]. Treatment of cells with Ac-DEVD.CHO protected them from CD95-induced apoptosis, but did not prevent the proteolysis of fodrin [128], suggesting that fodrin proteolysis can be uncoupled from apoptosis and that it is mediated by a caspase other than caspase-3. If correct, this is an intriguing observation, as it suggests that some caspases (those responsible for the cleavage of fodrin) may be activated and yet apoptosis may still be prevented. An alternative possibility is that Ac-DEVD.CHO may have inhibited only some features of the apoptotic phenotype, including those used to assess apoptosis in the particular study [128]. The inhibitory characteristics of the caspase responsible for fodrin proteolysis, i.e. readily inhibited by Ac-YVAD chloromethyl ketone and relatively insensitive to Ac-DEVD.CHO, are reminiscent of caspase-6.

Protein kinase C δ and Rb

Protein kinase C δ is also specifically cleaved during apoptosis to a catalytically active fragment by caspase-3, but not by caspases-2, -4, -5, -6 and -7. Protein kinase C δ is one of the few examples of a substrate that is cleaved by caspase-3 but not by caspase-7. Interestingly, overexpression of this fragment, but not of full-length protein kinase C δ or a kinase-inactive fragment, is associated with chromatin condensation, nuclear fragmentation and cell death, suggesting that the proteolytic activation of protein kinase C δ may contribute to certain features of the apoptotic phenotype [130,131].

Rb is an important mediator of cell cycle progression and regulation. Phosphorylation of Rb by cyclin-dependent kinases inactivates its growth-suppressive functions and drives cells through the cell cycle into mitosis. More recently, an anti-apoptotic function of Rb has also been described [132]. Several groups have described the cleavage of Rb during apoptosis [111,133,134]. Interestingly, different-sized cleavage products of Rb were observed in these studies, suggesting the possible involvement of different caspases. In one study, the cleavage site of Rb was identified as a DEAD↓G motif in the C-terminal region of the molecule, suggesting the likely involvement of caspase-3. The cleaved Rb failed to bind the regulatory protein Mdm2, which may lead to apoptosis due to inactivation of Rb functions [111].

INHIBITION OF CASPASES BY CrmA, Bcl-2 FAMILY MEMBERS, p35 AND PEPTIDE INHIBITORS

CrmA

When ectopically expressed, the *crmA* gene prevents apoptosis in a number of different systems [21,22,58,92,135–137] (Table 4). However, the ability of CrmA to inhibit apoptosis is clearly dependent on the stimulus used to induce it (Table 4). Thus there are CrmA-sensitive and -resistant pathways present in the same cell types. The recognition motif cleaved within CrmA is LVAD [138]. CrmA was originally described as a novel specific inhibitor of caspase-1 [45], but it is now apparent that it differentially inhibits different caspases. CrmA is a protease inhibitor that inhibits cell death, most probably by inhibiting one or more caspases. CrmA is a poor inhibitor of CED-3, caspase-2, caspase-3, caspase-7 and caspase-10, but an effective inhibitor of caspases-1, -4, -6 and -8 (Table 1) [58,65,67,83,84,135,138,139]. CrmA blocks both CD95- and TNFR-1-mediated cell death at much lower concentrations than are required for the inhibition of caspase-3 and caspase-7 [23,49,50,64,85]. Caspase-3 and caspase-7 are proteolytically activated following stimulation with CD95 or TNFR-1, but remain as zymogens in anti-CD95-treated CrmA-expressing cells, suggesting that CrmA inhibits a protease upstream of caspases-3 and -7 [85,140]. The effects on caspases-8 and -10 may be particularly important, as both of these caspases are capable of processing themselves and all other known caspases [67], and caspase-8 appears to be at the apex of the apoptotic cascade induced by CD95 or TNF [96,97]. Caspase-8 is very sensitive to inhibition by CrmA, whereas caspase-10 is only poorly inhibited, requiring a $\sim$1000-fold greater concentration of CrmA to give the same inhibition [67]. Thus the sensitivity of CD95-induced apoptosis to inhibition by CrmA appears to be due to inhibition of caspase-8. Overexpression of caspase-8 results in apoptosis, which is inhibited by CrmA, suggesting that caspase-8 may be the physiological target of CrmA or, alternatively, that there is a downstream CrmA-

Table 4 Bcl-2 and CrmA regulate different pathways

Apoptosis induced in the same cells by different stimuli is often CrmA-sensitive or CrmA-resistant. The former pathways are frequently Bcl-2/Bcl-x_L-resistant, while the latter are Bcl-2/Bcl-x_L-sensitive. Abbreviations: NGF, nerve growth factor; ND, not determined.; Ara-C, Cytarabine.

Treatment	Cells	CrmA-sensitive	Bcl-2/Bcl-x_L -sensitive	References
Mouse ICE	Rat-1	+++	+++	21
CED-3	Rat-1	+	+	21
Caspase-2	Rat-1, HeLa	+	+++	58,135
NGF withdrawal	Dorsal ganglion neurons	++	++	22
CD95	Jurkat, BJAB, MCF7	+++	–	135,140
Staurosporine	Jurkat T cells	–	+++	140
CD95	Lymphoma cells	+++	–	136
γ-Irradiation	Lymphoma cells	–	+++	136
CD95	U937	+++	ND	137
Ara-C	U937	–	+++	137
TRADD	HepG2, NIH 3T3	++	–	92

sensitive protease. These results further support the suggestion that caspase-8 lies at the apex of the apoptotic cascade triggered by CD95 and TNF, and explain why CrmA is such a potent inhibitor of apoptosis resulting from these stimuli (Figure 4). In contrast, CrmA-insensitive pathways may involve caspase-10 or a closely related homologue as their most upstream caspase (Figure 4).

Bcl-2 family

Bcl-2 is the mammalian homologue of CED-9, which is a negative regulator of CED-3 [19]. Bcl-2 and related family members, such as Bcl-x_L, inhibit cell death induced by many stimuli [141,142]. Interestingly, many of the apoptotic pathways that are sensitive to inhibition by CrmA are relatively resistant to Bcl-2/Bcl-x_L, and vice versa (Table 4). These findings suggest the presence of a CrmA-sensitive (CD95)/Bcl-x_L-insensitive and a CrmA-insensitive/Bcl-x_L-sensitive pathway for the induction of apoptosis [137]. As discussed earlier, specific interactions of TRADD and FADD with an intracellular domain of TNFR-1 and CD95 respectively induce apoptosis, and both of these are inhibited by CrmA, whereas neither Bcl-2 nor the *E1B* gene product block TRADD-induced cell death [91,92]. The CrmA-sensitive pathways generally appear to involve plasma membrane receptor-mediated apoptosis, compatible with the inhibition of a specific caspase (such as caspase-8) being recruited to the cell membrane. Bcl-2/Bcl-x_L seems relatively ineffective at inhibiting this type of receptor-mediated apoptosis, but more efficacious than CrmA at inhibiting other forms of apoptosis.

Several recent studies suggest that Bcl-2 and Bcl-x_L exert their anti-apoptotic action at or before the processing of certain caspases to their catalytically active forms [140,143–145]. For example, overexpression of Bcl-2 or Bcl-x_L prevents staurosporine-induced cell death of Jurkat T cells and the processing of both caspase-3 and caspase-7, placing these negative regulators of apoptosis at or upstream of the processing of caspases-3 and -7 [140].

p35

The baculovirus *Autographa californica p35* gene product inhibits apoptosis in insects, mammals and nematodes, suggesting that it acts at a central and evolutionarily conserved part of the apoptotic pathway [24,138,146,147]. Purified recombinant p35 inhibits the activity of purified recombinant caspases, including caspases-1, -2, -3 and -4, and maximum inhibition is achieved at equimolar concentrations of p35 and the caspase. Cleavage of p35 by a caspase results in the formation of a caspase–p35 complex. The presence of such complexes *in vivo* would prevent the caspases from initiating the apoptotic cascade. The CED-3 cleavage site in p35 is DQMD↓G, which is required for protection against programmed cell death in the nematode. Interestingly, CrmA does not protect against cell death in the nematode unless its caspase-1 cleavage site (LVADC) is replaced by the CED-3 cleavage site in p35 [138].

Peptide inhibitors

In addition to the reversible inhibitors Ac-DEVD.CHO and Ac-YVAD.CHO, use of the irreversible tripeptide caspase inhibitor Z-VAD.FMK [benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone] has helped to elucidate the role of caspases in apoptosis, in particular in intact cellular systems. Z-VAD.FMK is a cell-permeable irreversible inhibitor of caspases whose permeability is facilitated by the presence of the benzyloxycarbonyl and OMe groups. Z-VAD.FMK is a potent inhibitor of apoptosis induced by a wide range of stimuli in a number of different systems, including thymocytes, hepatocytes, human monocytic THP.1 cells, Jurkat T cells, neuronal cells and *Drosophila melanogaster* [72,144,148–153]. Z-VAD.FMK also suppresses programmed cell death in the interdigital webs of developing mouse paws, suggesting that it may be of value in studying developmental cell death [72,154]. Z-VAD.FMK inhibits apoptosis at an early stage, as judged by its inhibition of all the ultrastructural features of apoptosis, i.e. PARP cleavage, processing of caspase-3, formation of large fragments and internucleosomal cleavage of DNA [71,72,149,150]. More recently we have shown that it inhibits the processing of caspases-2, -3, -6 and -7, suggesting that it inhibits a caspase at or near the apex of the apoptotic cascade [62]. In this regard, it is of interest that Z-VAD.FMK blocks apoptosis induced by caspase-8 [97]. Many, but not all, apoptotic cell deaths are inhibited by Z-VAD.FMK [155,156]. Whereas it completely inhibited apoptotic death induced by different stimuli in thymocytes [150,155], it had a variable effect on cell death in peripheral T cell blasts while complete sensitivity to another caspase inhibitor was maintained, suggesting the activation of different caspases at different stages of T-cell maturation [155]. Z-VAD.FMK did not inhibit Bax (a Bct2 family member)-induced cell death, although it did inhibit certain features of the apoptotic phenotype, including cleavage of nuclear and cytosolic substrates and DNA fragmentation, suggesting that Bax-induced cell death may not require caspases [156]. *In vivo*, Z-VAD.FMK prevents the normally fatal liver damage induced by anti-CD95 injection [157]. Activation of both caspase-1-like and caspase-3-like protease activities is detected in liver lysates, as is the cleavage of caspase-3 to its p17 fragment. Repeated injections of Z-VAD.FMK results in the complete survival of all animals, with no histopathological signs of liver damage. The treatment with Z-VAD.FMK does not prevent the initial early small rise in ICE-like activity, as assessed by Z-YVAD-T-amino-4-trifluoromethylcoumarin cleavage; however, it does prevent processing of caspase-3 and the increase in caspase-3-like protease activity, as assessed by Z-DEVD-T-amino-4-trifluoromethylcoumarin cleavage. This and other observations suggest that non-toxic caspase inhibitors may have potential clinical applications in fulminant hepatitis arising from viral hepatitis [157] and various neurodegenerative disorders [5,154].

A HIERARCHY OF CASPASES

All caspases are cleaved at specific Asp residues, raising the possibility that some caspases sequentially activate others, so establishing a hierarchy of caspases. Such a model has been proposed in which caspase-8 has been termed an 'initiator' protease, which activates an 'amplifier' protease such as caspase-1, which in turn activates a 'machinery' protease such as caspase-3 or caspase-7 [158]. Much of the evidence for this, and the concept of a hierarchy of caspases, is based on *in vitro* data with recombinant enzymes; the limitations of such approaches have already been discussed. It is important, where possible, to determine if such a cascade of caspases occurs in cells undergoing apoptosis. PARP cleavage generally precedes lamin cleavage in cells undergoing apoptosis [68,87,115,116]. As caspase-3 and caspase-7 cleave PARP but not lamins, and caspase-6 cleaves lamins, this suggests that, in cells undergoing apoptosis, activation of caspase-3 and caspase-7 precedes activation of caspase-6 (Figure 4). In human monocytic tumour cells undergoing apoptosis, processing of caspases-2, -3, -6 and -7 was observed, although the precise sequence of activation of these caspases was not determined [62].

Different structural features, such as specific cleavage-site motifs, in the different caspases may give clues to their activities and to which caspase lies upstream of others (Table 1; Figures 2 and 3). The presence of a DXXD motif in caspases-2, -6 and -9 (Table 1) suggested that these three caspases may be activated by caspase-3, and this has been substantiated by *in vitro* experiments. A WXXD motif occurs in all three members of the ICE subfamily (Table 1), suggesting that they may be activated by the same caspase. Similarly, IXXD and LXXD motifs recur in different caspases (Table 1), supporting the notion that there may be a small number of key caspases that activate other family members. Good candidates for such critical caspases are caspase-8 and caspase-10, because they activate all other caspases and also contain FADD-like prodomains, which permit extensive protein–protein interactions [66,67,96,97]. Stimulation with either CD95 or TNF results in the recruitment and activation of caspase-8, supporting the hypothesis that it lies at the apex of the apoptotic cascade following specific receptor-mediated activation (Figure 4). Other types of receptor-mediated activation may involve other caspases, such as caspase-10. It has been proposed that caspase-10 may be involved in many CrmA-insensitive non-receptor-mediated cell deaths, as it is poorly inhibited by CrmA [67]. Once activated, some family members may then further activate the caspase that initially caused their activation, so setting up a caspase amplification cycle (Figure 4) [82]. The presence of CrmA-sensitive and -insensitive pathways also suggests that not all cell death pathways utilize the same caspases.

While many intracellular targets of the caspases have been recognized (Table 3), the critical cellular substrates leading to cell death have not yet been identified. The mechanisms by which caspases are regulated in cells in order to prevent their unwanted demise, and the possible role of apoptosis-inhibitory proteins, will be the subject of intense investigation. The long prodomains of some caspases, such as caspase-2, may enable them to be recruited by adapter molecules such as RAIDD/CRADD, so facilitating the execution of the cell death programme [101,102]. It remains to be determined whether there is a family of RAIDD/CRADD-like molecules that can recruit other caspases with prodomains.

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